Manuscript Details

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Title	Heterogeneity and coexistence of oncogenic mechanisms involved in HCV-associated B-cell lymphomas
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Abstract

The association of HCV-infection with B-lymphomas is supported by the regression of most indolent/low-grade lymphomas following anti-viral therapy. Studies on direct and indirect oncogenic mechanisms have elucidated the pathogenesis of HCV-associated B-lymphoma subtypes. These include B-lymphocyte proliferation and sustained clonal expansion by HCV-envelope protein stimulation of B-cell receptors, and prolonged HCV-infected B-cell growth by overexpression of an anti-apoptotic BCL-2 oncogene caused by the increased frequency of t(14;18) chromosomal translocations in follicular lymphomas. HCV has been implicated in lymphomagenesis by a "hit-and-run" mechanism, inducing enhanced mutation rate in immunoglobulins and anti-oncogenes favoring immune escape, due to permanent genetic damage by double-strand DNA-breaks. More direct oncogenic mechanisms have been identified in cytokines and chemokines in relation to NS3 and Core expression, particularly in diffuse large B-cell lymphoma. By reviewing genetic alterations and disrupted signaling pathways, we intend to highlight how mutually non-contrasting mechanisms cooperate with environmental factors towards progression of HCV-lymphoma.

Keywords	non-Hodgkin B-lymphoma; lymphomagenesis; hepatitis C virus; pathogenesis; mixed cryoglobulinemia; oncogenic pathways; genetic damage; chromosomal aberration
Manuscript category	Hematology

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To: Editor

Journal: *Critical Reviews in Oncology / Hematology*

Rome April 2, 2019

Dear Editor,

Enclosed, please find our revised manuscript (Ref: CROH_2018_387) entitled: "**Heterogeneity and coexistence of oncogenic mechanisms involved in HCV-associated B-cell lymphomas**".
Thank you for allowing our paper receive positive encouraging remarks and useful criticisms from the reviewers.
We have considered all issues mentioned in the reviewers' comments and made the requested changes in the manuscript.

- ✓ We reduced our review without giving up its purpose: to be omni-inclusive. The following paragraphs have been reduced of approximately 4 pages/2000 words:
  - PATHOGENESIS OF HCV-LYMPHOMA: WHICH MECHANISMS ARE INVOLVED?
  - THE IMPACT OF THE MICROENVIRONMENT AND miRNAs ON HCV-LYMPHOMAGENESIS
  - ANIMAL MODELS TO EXPLAIN HCV- LYMPHOMAGENESIS AND WHAT WE CAN LEARN
  - CONCLUSIONS
- ✓ Now, the speculative parts are mostly limited to the conclusions.
- ✓ To make the review more readable even to a wider range of non-biochemist/molecular biologist readers, the manuscript has been amended in a more didactic way. To increase its clarity, the paragraph "LYMPHOPATHOGENESIS RELATED TO THE HCV-LYMPHOTROPISM" has been further subdivided into the following subsections: Type II Mixed cryoglobulinemia; Evidence for HCV-lymphotropism; The role of HCV quasi-species; The involvement of HCV-cell entry.
- ✓ The combined treatment with DAA and appropriate chemotherapy in patients with nonHogdkin lymphoma and hepatitis C infection (Persico et al, Hepatology 2018) has been mentioned in the paragraph "HCV INVOLVEMENT IN LYMPHOMAGENESIS: MORE THAN A SIMPLE SUSPICION"
- ✓ The review from Costas L et al. (Crit Rev Oncol Hematol. 2014) has been cited in the paragraph "PATHOGENESIS OF HCV-LYMPHOMA: WHICH MECHANISMS ARE INVOLVED?"

We are confident that the current version is now suitable for publication in *Critical Reviews in Oncology / Hematology* Journal.

Thank you for your attention

Best wishes and kind regards,

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Response to reviewers:

-Reviewer 1

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-Editor

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INTRODUCTION

The hepatitis C virus (HCV), a member of the *Hepacivirus genus* of the *Flaviviridae* family, is a major cause for acute and chronic hepatitis worldwide, thus representing a major global health challenge [1]. Roughly 75% of HCV-infected subjects do not resolve acute infection and develop chronic hepatitis, an insidious condition that, in addition to causing steatosis and possibly liver failure [2], might evolve through fibrosis/cirrhosis into hepatocellular carcinoma (HCC) [3].

A deficit in host immune surveillance concerning humoral and cellular immunity, characterized by reduced cytotoxic T-lymphocytes (CTL), was shown to impair CD4+/CD8+ T-cell proliferation capacity and effector cell function [4, 5] allowing immune escape for the HCV and persistent viremia in chronic hepatitis C [6, 7]. Currently, despite the lack of an effective vaccine, direct-acting antiviral drugs (DAAs), alone or in combination, are able to cause viral clearance in most individuals infected with the major HCV genotypes [8, 9].

HCV-related hepatocarcinogenesis had not been fully clarified. This was ascribed to the inadequacy of *in vitro* models of HCV-infection, investigated to understand how the main oncogenic pathways of proliferation, regeneration and apoptosis, are modulated by the HCV in the infected hepatocytes [10]. Failure to identify all genetic/epigenetic alterations in oncogenes/anti-oncogenes and key genes in liver differentiation and angiogenesis, delayed understanding the mechanisms that drive transition of the HCV-infected hepatocytes from inflammation to malignant transformation, *via* fibrosis/cirrhosis. HCV chronic infection may also progress through some lymphoproliferative and autoimmune diseases, characterized by extrahepatic immune manifestations, which present different grades of aggressivity [11-13]. They encompass type II mixed cryoglobulinemia (MC) and monoclonal gammopathy of undetermined significance (MGUS) up to B-cell non-Hodgkin's lymphoma (B-cell NHL), reviewed in [14].

Increasing evidence, both *in vivo* and *in vitro,* for HCV lymphotropism [15-17] supports a direct involvement of HCV in lymphoproliferative disorders (LPDs) [18]. The primary pathogenic role of HCV in B-cell NHL was highlighted by the high prevalence of HCV infection in sera and replication in circulating lymphocytes from chronic carriers with MC and low-grade B-cell NHLs [11], as well as by efficacy of anti-viral therapy in eradicating most B-cell NHLs by viral load suppression after sustained virologic response (SVR), and by lymphoma relapse preceded by recurrence of viral load [19-21]. Unlike HCV-hepatocarcinogenesis [10], the heterogeneity of a wide range of LPDs that arise in chronic hepatitis C, their long development time and the large number of dissimilar pathologies and associated clinical evidence [13, 14, 18, 20], have prevented the formulation of a single, coherent model in HCV-lymphomagenesis.

Here we undertook a comparative analysis of the most common steps and oncogenic patterns, which can present synergy in the development of B-cell NHLs, by reviewing clinical and experimental studies, which have allowed elucidation of the main molecular pathways and viral/host factors involved in HCV-lymphomagenesis.

CELLULAR AND MOLECULAR TARGETS INVOLVED IN HCV-REPLICATION IN THE HOST

The HCV genome, a positive-strand (+) RNA of about 9,600 nucleotides (nt) [22] is composed of a long open reading frame of 9,024 nt, coding for a single polyprotein precursor of 3,000 amino acids (aa), flanked at the 5' and 3' ends by two highly conserved untranslated regions

(UTRs), required for viral RNA translation and replication. The 5' UTR is a non-coding region of 341 nt, containing an internal ribosome entry site (IRES) for translation initiation of a single polyprotein, cleaved in 10 proteins [23] by cellular and viral proteases. In the 5' UTR, interaction sites with miR-122 are located, upstream of IRES, which act as positive regulatory factors on RNA replication, stimulating translation and enhancing viral RNA replication. Downstream of the 5' UTR, several cis-acting RNA elements (CREs) are also positioned that, together with two stem loops in the core region, stimulate HCV RNA translation. Core and E1-E2 envelope glycoproteins are the major structural proteins of viral particles, and function as ligands for cellular receptors. The other seven proteins are nonstructural (NS) proteins. NS2, acting as a cysteine protease, is required for the NS2-NS3 junction cleavage generated by E2 cleavage that, together with the p7 viroporin protein, is involved in virus assembly. The five others NS proteins participate in the replicase complex: NS3 contains serine protease activity in the N-terminal domain, activated by the NS4A cofactor, while in its C-terminal there are NTPase and helicase activities required for RNA replication; NS4B triggers membrane rearrangement associated with the viral replication complex [24]; highly phosphorylated NS5A is involved in RNA binding, replication and assembly of HCV; NS5B functioning as a RNA-dependent RNA polymerase (RdRp) is the HCV replicase.

At the 3' end of 3' UTR a regulatory region of 200-235 nt is located, including a short variable region, a poly-U/UC tract of about 80 nt and the X-tail; a conserved RNA region of 98 nt that, along with the enclosed minimum poly (U) tract of about 25 nt, is involved in RNA replication [23]. The synthesis of the minus (-) strand HCV RNA, functioning as a replication intermediate (RI), starts at the 3' non-coding region (NCR) end of (+) strand genomic RNA. This mode of HCV RNA replication with (-) strand RNA serving as a template for producing a large excess of (+) strand RNA has been reported as asymmetric and semiconservative in others Flavoviruses. A recent model to explain regulation of the HCV RNA translation/replication switch has been proposed based on viral RNA circularization through interactions between IRES motifs in the 5' UTR and stem-loop structure at the 3' end in the NS5B coding region [25]. Overall, HCV RNA replication appears as a multistep process coordinated by viral and cellular proteins, and requires extensive intracellular membrane remodeling for biogenesis of replication factory and also exploits components of the intracellular lipid transport system for producing infectious virions [23].

Since the discovery of HCV by cDNA cloning identification of the virus genome [22], HCV molecular studies using HCV-recombinant clones and chimeric constructs have been conducted in *in vitro* cell models allowing efficient HCV-infection, replication and very high production levels of different virus genotypes, which can be expanded to different host cells [23, 24, 26-29]. In the last two decades, this approach has enabled a dramatic breakthrough in HCV molecular biology, unveiling virus coding capacity, polyprotein processing, protein functions, encapsidation, assembly and molecular targets in HCV replication [29-31] for contributing to developing effective antiviral agents and vaccines [9, 27].

Adaptation of robust cell systems for *in vitro* HCV infection has allowed the investigation of the molecular and enzymatic mechanisms of HCV RNA replication and has helped in clarify the main stages of the HCV life-cycle [23-29]. This has enabled the major progress in the basic knowledge of HCV entry and some clinical implications of infection [31].

However, the experimental strategy based on synthetic models [26, 27], which combines cell systems and surrogate infectious viruses not found in nature [32], has not greatly helped in understanding HCV pathobiology [17]. One of the main characteristics of native HCV RNA has not

been considered, i.e. to be present *in circulum* in the form of viral variants, called quasi-species, involving more than seven major genotypes and many more subtypes. Viral quasi-species (SVP) are due to the extraordinary genetic variability of HCV, ascribed to the high virus mutation rate caused by the error-prone mechanism of RNA polymerase [27] and these variants contribute to enlarging the viral host-spectrum.

THE DOUBLE HEPATIC AND LYMPHOID TROPISM OF THE HCV

Since the discovery of HCV as the etiological agent responsible for non-A and non-B hepatitis [22], HCV has also been found closely associated with a number of benign and malignant lymphoproliferative and autoimmune regulated diseases, principally the MC and B-cell NHL, mostly in countries with high prevalence of viral infection, on the basis of clinical-epidemiological surveys [11-14, 17-20].

Confirmation of the dual HCV-pathogenesis was achieved in extrahepatic and hepatic infected tissues investigating for HCV-virions by electron microscopy (EM) or immune-EM, for the presence of (+) and (-) HCV RNA strands by *in situ* hybridization (ISH) or highly strand-specific RT-PCR, using rTrh DNA polymerase to avoid or reduce false positivity [33], and for the expression of viral proteins by immunohistochemistry (IHC) or immunofluorescence (IF) [17].

The efforts to obtain HCV productive infection *in vitro* were firstly achieved by using the serum derived to inoculate primary fetal and adult differentiated hepatocytes [17, 34-38] or infecting hepatoma cell line-derived hepatic cells by recombinant/mutated HCV-strains, such as HCVcc and HCVpp cell systems [26-28]. Hepatic progenitor cells from human embryos have been found to be permissive to HCV infection and persistently produced the virus, whereas pluripotent stem cells were not permissive, with permissiveness dependent on liver specific miR-122 expression and cell factors involved in HCV replication [39].

The initial difficulties faced for detecting strong and persistent HCV replication in *in vivo* infected livers [40] and *in vitro* inoculated liver cell systems, using semi-quantitative and quantitative RT-PCR, privileged the search for virus lymphotropism by localizing HCV in lymphoid cells of extrahepatic districts in HCV-infected subjects and attempting to propagate the virus in cells of hematopoietic origin [17, 27]. Hence, over the last two decades, the pathogenic role played by the hepatic and lymphoid tropism of HCV has been widely investigated, not only in the establishment of persistent infection, but also in the induction of chronic inflammation and immune stimulation that may lead to hepatocarcinogenesis and LPDs and B-cell NHLs in lymphoid districts.

LYMPHOPATHOGENESIS RELATED TO HCV-LYMPHOTROPISM

The polymorphism of clinical manifestations belonging to the so-called "HCV syndrome" has been recently described in detail with particular emphasis on specific morpho-histopathological features and extrahepatic tissue localizations in relation to the diverse clinical courses of chronic hepatitis C preceding the evolution toward MC and B-cell NHLs [13, 20].

Type II Mixed cryoglobulinemia is characterized by an excessive production of autoantibodies and immune complexes in the serum, including mixed cryoglobulins (a mixture of different immunoglobulins (Igs), that can precipitate, becoming insoluble at low temperatures [12, 20]), whose monoclonal component is often associated with IgM with rheumatoid factor (IgM-RF)

activity and HCV RNA. Mixed cryoglobulins result from HCV-host interactions, which are dependent on numerous cofactors for pathogenicity. MC is a process characterized by deposition of circulating cryoglobulins on small vessels and is also called cryoglobulinemic vasculitis. MC is associated with a potential risk of developing B-cell NHL in about 10% of MC patients [13, 14].

MC defines HCV-driven immune mediated and lymphoproliferative disorders, followed by poly-oligo clonal B-lymphocyte expansion able to evolve into frank malignant lymphoma. The presence of lymphoid follicles in liver, mostly in cases of MC, similar to those found in HCV infection, indicates that these are sites of expansion of clonal B-cell, which also secrete RF [14]. Antigen-driven stimulation in HCV-related LPDs was demonstrated by the presence in the same patient of mutations in Ig variable heavy V(H) and variable kappa light V(K) genes of B-cell clones in premalignant and malignant lymphoproliferative manifestations, therefore representing sequential phases of the same antigen-driven pathological process [41].

Longitudinal analysis of immunoglobulin chain (IgH) mutations and expression of Ig antigen receptors, displaying sufficient homology with an anti-HCV protein, strongly confirmed that MC and the subsequent B-cell NHL were both antigen-driven LPD processes closely dependent on HCV infection. Therefore, MC can be considered a systemic autoimmune condition, characterized by cutaneous and organic involvement, which may be associated with chronic HCV-infection [20]. The hepatic involvement in MC, less frequent in systemic vasculitis, strongly suggests a hepatotropic role for HCV in this process. In particular, analogously in B-cell NHLs, MC mimics some immune-mediated disorders and malignancies, while demonstrating a pathogenic role of chronic HCV-infection in various MC forms associated with extrahepatic manifestations [13, 20]. These include rheumatic diseases and endocrine disorders, involving the thyroid, development of diabetes and, associated with sexual dysfunctions, a form of skin porphyria and a severe renal involvement of unfavorable prognosis.

Undeniably, B-cell clonal expansion characterizing MC seems to be the product of dysregulation of the immune system rather than the result of a true hematological malignancy [42]; several viral and host factors are required for MC development.

Evidence for HCV-lymphotropism. HCV lymphotropism has been clearly demonstrated [16-18], and represents a major advance in understanding the pathogenesis of HCV-associated B-cell LPDs leading to B-cell NHL. HCV replication was firstly observed in peripheral blood mononuclear cells (PBMCs) from *in vivo* HCV-infected patients with chronic hepatitis [14, 16]; HCV replication was also observed in MC/B-cell NHL [11, 43], in patients coinfected by HCV and HIV, and in some forms of occult HCV-infection [17], as well as in B-/T lymphocytes, macrophages, Kupffer cells, bone-marrow (BM) cells and dendrocytes [17, 44-49]. Furthermore, a virus inoculum derived from HCV-infected sera and other *in vitro* sources, was able to infect lymphoid cell cultures [50-54]. However, few systems for long-term culture of HCV have been obtained [17] by de *novo* and *in vitro* HCV-infection of B and T cells [54-56] or culture of B-cell lines persistently producing the infectious virus, derived from an HCV-positive lymphoma and presenting enhanced apoptosis [53]. This has important implications in HCV pathogenesis, demonstrating a cell-free virus sub-passage from HCV-infected macrophages to B-cells or hepatocytes [56], and also direct cell-to-cell HCV transmission from persistently HCV-infected human BM-derived lymphoblastoid cells to recipient hepatoblastoma cells [57] and by exosomes [58].

The reported evidences emphasized the relevance of the lymphoid compartment in establishing and propagating HCV infection, see [17] for review. However, conspicuous and

continuous virus production was unsuccessful by inoculating *in vitro* human B and T cells with wild-type virus strains, or using the JFH1-strain of HCV, which binds to but fails to infect B-cells [59]. There are many clinical and experimental investigations, which strongly confirm the existence of lymphotropism determinants in HCV proteins.

The role of HCV quasi-species. Increasing evidence has shown that HCV circulates in the form of heterogeneous RNA quasi-species, caused by high error-prone HCV RNA synthesis [60]. This has enabled better understanding of the extraordinary host-adaptation and variability that characterize some HCV variants [17]; it could be hypothesized that RNA quasi-species allow the virus to evade clearance by T and B lymphocyte immunity, leading to persistent infection in most infected subjects [61] as in the case of viral variants in the hypervariable region 1 (HVR1) of E2-envelope glycoprotein, under selective immunological pressure. Specific circulating viral quasi-species were firstly found compartmentalized in B-cells and monocytes [62] indicating the lymphoid district as a unique "*virus reservoir*" of the host-infected organism. Mutations specifically located in the 5' UTR of the HCV hepatotropic strain H77 enhance virus replication when grown in T lymphoid cells of the MOLT-4 cell line [52]. Thus, the presence of strain-specific sequences in the 5' UTR or different sequence heterogeneities in the E1 and E2 coding regions, can give rise to altered lymphotropism relative to hepatotropic virus strains [62, 63]. A quasi-species compartmentalization, observed in PBMCs from transplant recipients, strongly suggests that lymphoid tissues may also hold variants more susceptible to infecting lymphoid cells [64]. Compelling data has enabled identification of genetic determinants, within E1/E2, related to the entry lymphotropism, which in HCV chronic-infected HCV patients may convert the virus from hepatotropic to lymphotropic [65]. Indeed, a co-receptor B7-2 (CD86) was identified specific for infection by lymphotropic HCV in an HCV-positive B-cell lymphoma; this receptor was shown to mediate HCV infection of memory B-cells, leading to inhibition of their function and enhancing their differentiation into IgM-secreting plasmablasts [66]. Silencing of HCV replication in B-cells is promoted by virus sensor retinoic acid-inducible gene I (RIG-I) or microRNA-122 overexpression. Despite its low rate, the genetic recombination in HCV may also contribute to the observed virus genetic variations and display some pathogenic and clinical implications by generating escape mutants that confer drug resistance [67].

The involvement of HCV-cell entry. Before undergoing clathrin-mediated endocytosis and membrane fusion in the host-cell endosome, HCV enters the cells by interaction of the viral particle with the host-cell membrane. This involves prior attachment of E1 and E2 viral envelope glycoproteins with several cell cofactors, through a process involving extremely adjusted steps [31]. The HCV entry process requires glycosaminoglycans (GAGs) and low density lipoprotein receptors (LDL-R) for virus adhesion to the cell surface; then the tetraspanin CD81 (the first to be identified among the principal cell receptors/co-receptors), the high density lipoprotein receptor SR-B1, the two tight junction (TJ) proteins, claudin-1(CLND1) and occludin (OCLN), the Niemann-Pick C1-like 1 cholesterol adsorption receptor and the transferrin receptor 1 (TfR1) [68, 69]. Of these co-receptors, which are associated with the E1 and E2 HCV envelope proteins, CD81 binds the virus with a high affinity [70, 71], and appears to be the most ubiquitous, since it is overexpressed in B lymphocytes, as well as in hepatocytes and a number of different cells. This means that persistent stimulation of lymphocytes by viral surface antigens may be primarily implicated in the development of B-cell LPDs, such as MC and benign and malignant B-cell NHLs, see [14] for review. In fact, continuous antigenic stimulation of the HCV-induced B cell immunological response may lead to an initial oligo-polyclonal B-cell expansion, causing further genetic aberrations and mutations [71-73].

To explore the causal relationship between HCV infection and B-cell NHLs, environmental and genetic/epigenetic alterations, occurring in oncogenes and anti-oncogenes by only partially known mechanisms, have been investigated in-depth [14, 18, 20]. The subsequent clonal expansion of B cells following HCV infection may induce genetic modifications and alterations of signaling pathways involved in cell growth and survival leading to lymphoid cell transformation, which characterizes most of the current models in HCV-lymphomagenesis.

HCV INVOLVEMENT IN LYMPHOMAGENESIS: MORE THAN A SIMPLE SUSPICION

A number of epidemiological studies, recently reviewed [18, 19, 73], have supported the notion that HCV infection is a risk factor for B-cell NHL, playing a primary role in the development of premalignant and malignant lymphoproliferative diseases.

From meta-analysis of case-control clinical-epidemiological studies, the relative risk (RR), globally, of being infected by HCV was shown not to exceed a factor of 2.4 among patients with B-cell lymphoma relative to the general population [73]; HCV prevalence in B-cell NHL patients was about 15% respect to the controls [19]. The fraction of B-cell NHLs secondary to HCV-infection can reach 10-15% or even more in areas of high prevalence of HCV infection, like Italy and Japan, but decreases significantly in areas of low prevalence [18, 73]. About 5-10 % of benign MC progresses to lymphoma, while in HCV-infected patients with MC, the risk of developing B-cell NHL increases by 35-fold [74].

Most epidemiological studies addressed at establishing a causal relationship between chronic HCV-infection and B-cell lymphomas have mostly been inconclusive, especially when conducted in areas with low HCV prevalence, as well as studies that attempted to associate lymphoma risk with its histological subtype and HCV genotype [18, 19]. Antiviral treatment of HCV with interferon (INF) and ribavirin (RBV), demonstrated the regression of splenic lymphoma with villous lymphocytes [21]; this initial result was followed by numerous clinical trials that were successful in eradicating most indolent low-grade B-cell NHLs and MC by curing HCV, thus indicating a close causal link with HCV [19, 75].

A recent study enrolled a very wide homogeneous population (HCV cohort of more than 10,000 carriers and a non-HCV population of more than 40,000) and was followed-up for 8 years to minimize the inadequate temporal length, which in most of the studies reported may have considerably affected the NHL risk prevalence [76]; this study observed that the incidence rate of any type of lymphoid neoplasms, including B-cell NHLs (75%), was significantly greater ($P < 0.0001$) in the HCV cohort than the non-HCV cohort, 48.4 versus 22.1 per 100,000 person-years.

The three main histological subtypes frequently associated with HCV infection are diffuse large B-cell lymphomas (DLBCL), marginal zone lymphomas (MZL) and lymphoplasmacytic lymphomas (LPL) [77]. In a European multicenter case-control study (EPILYMPH), patients displaying HCV RNA positivity where associated with a greater risk of DLBCL. DLBLC, the most widespread large B-cell NH lymphoma in western countries, is also the most aggressive lymphoma, and there is a significant association between HCV infection and B-cell NHL. When associated with HCV, DLBCL displays an aggressive clinical form, which requires combined immune-chemotherapy with rituximab (anti-CD20) plus cyclophosphamide, vincristine, prednisone and doxorubicin (CHOP-R) [18, 73, 78]. Conversely, for HCV-associated indolent B-cell NHLs, the traditional

antiviral therapy (INF + RBV) was effective to achieve HCV eradication, and was followed by lymphoma regression in the majority of cases, reaching 75% in MZL [18].

IFN-free DAAs, as a first-line treatment for HCV, are effective in curing HCV in around 98% of chronic carriers harboring indolent B-cell NHLs [79]. The overall hematological response rate was 67% of cases (26% presented complete lymphoma remission). Of the MZL patients, the highest rate of lymphoma response was 73%. Therefore, particularly for patients with HCV-associated indolent lymphoma of this subtype, DAAs are a very promising safe antiviral treatment, while, for complete remission of patients with aggressive lymphoma, the DAA treatment required additional chemotherapy to be effective [80].

PATHOGENESIS OF HCV-LYMPHOMA: WHICH MECHANISMS ARE INVOLVED?

Causal agents of B-cell NHLs can be genetic factors [81] or acquired factors, namely environmental and infectious agents such as lymphotropic viruses: Epstein-Barr virus (EBV), human immunodeficiency virus (HIV), HTLV, herpes virus 8 and more recently also the HCV and the bacterium *Helicobacter pylori*, reviewed in [18, 73]. Among the risk factors that can induce host immunodeficiency favoring lymphoma development, reproductive factors such as hormones, have been recognized [82]. Other unidentified host-factors also play a key role, as suggested by the heterogeneity of histology, localization and clinical course of lymphomas related to infectious agents, or the discrepancy between the high prevalence of (often ubiquitous) infectious agents such as the EBV in B-cells of the infected carriers relative to the low percentage of patients developing lymphoma. In particular, it was recently hypothesized that each of the individual etiological agents that provide low oncogenic signals, are not strong enough to cause lymphoma alone, but may cooperate to produce a composite stimulus sufficient to give rise to a lymphoma [83].

Epidemiologists have suggested that developing B-cell NHL is a multi-step and multi-causal event, due to different etiologic mechanisms that can be mutually non-exclusive [83]. In fact, HCV-induced lymphomagenesis may also result from various oncogenic mechanisms [72, 81], acting through multi-stage and multi-factorial processes that may cooperate to develop a lymphoma [20, 84]. Experimental data [11, 53], has provided strong evidence for an oncogenic role of the HCV genome and/or of some of its viral proteins in infected B lymphocytes, reviewed in [85]. However, the role of virus interaction, penetration and replication in B-lymphocytes still remains to be fully clarified; the observed association of HCV with increased autoantibodies in MC and B-cell NHLs, and increased peripheral innate CD5+ B cells have suggested a pathogenic role for B cells in viral specific activation and clonal proliferation characterizing HCV-infection [84, 86]. Furthermore, convincing evidence has recently supported the fact that CD5+ cells only mediate HCV-infection in T-lymphocytes, without developing lymphoma [87].

Multistep processes allowing development of current models on lymphomagenesis associated with HCV, as outlined in Figure 1, include: a) HCV-induced chronic B-cell immune-stimulation leading to lymphoma; b) chromosomal aberration in HCV-associated lymphomagenesis; c) a direct HCV-infection of B-lymphocytes produces permanent genetic damage; d) direct oncogenic role of HCV in B-lymphocytes; e) signaling pathways potentially involved in HCV-lymphomagenesis; f) B-cell lymphoproliferation mediated by cytokines and chemokines.

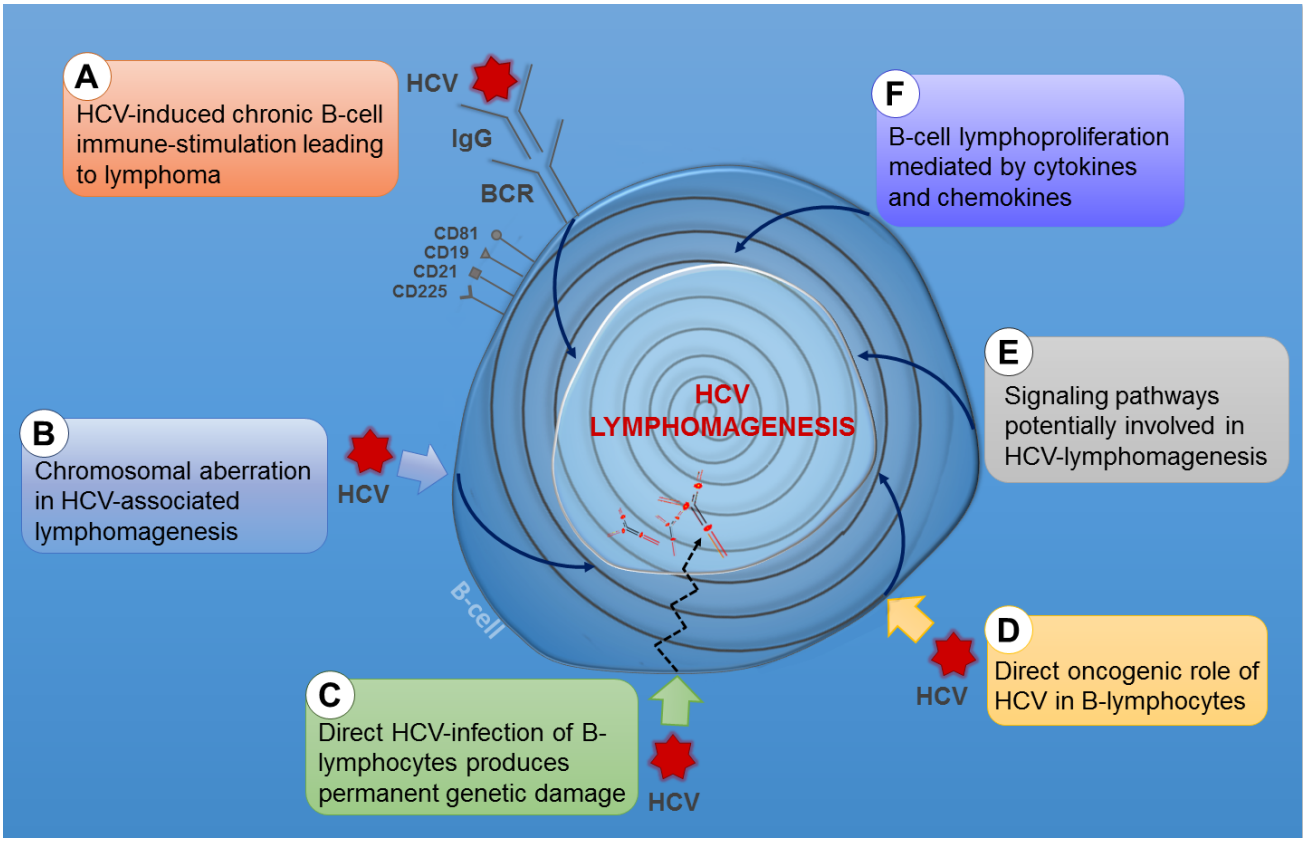

Figure 1. Current models of HCV-associated lymphomagenesis. (A) External antigenic stimulation of HCV-surface antigen binding CD81 and BCR receptors by HCV-envelope proteins induces proliferation and sustained clonal expansion of B-lymphocytes leading to MC and frank B-cell NHL. E2-CD81 binding enables the B-cell costimulatory complex by TK phosphorylation and Ig gene hyper-mutation. E2-CD81 signaling induces immune escape and LPD by an increase in costimulatory CD80/CD86 and a decrease in the complement receptor CD21. **(B)** Overexpression of anti-apoptotic *BCL-2* oncogene by increased frequency of chromosomal t(14;18) translocation prolongs HCV-infected B-cell growth preventing apoptosis, due to alteration of the BCL-2/BAX ratio. **(C)** HCV has been implicated in lymphomagenesis by a "hit-and-run" mechanism, inducing hyper-mutations of Igs in infected B-cells, and oncogenes/anti-oncogenes through permanent genetic damage, caused by DNA double-strand breaks inducing error-prone DNA polymerase by activation-induced cytidine deaminase that stimulates TNF-α production. **(D)** More direct oncogenic mechanisms have been explored, such as the role of the intracellular viral proteins as Core and NS proteins. HCV Core protein is required for cell growth and proliferation, upregulating TP53 family isoform protein DNp63 transcription and enhancing TNF-α induced apoptosis. NS3 + Core induces *in vitro* NOS and ROS, causing DNA repair damage and is closely associated with high-grade lymphomas *in vivo*. NS5A protects from TNF-α and TP53-induced apoptosis and promotes tumor growth. Core and NS5A behave as transcriptional trans-activators for several genes. NS4B modulates NS5A hyperphosphorylation and transactivates IL-8, inducing an unfolded protein response and ER overload response-dependent NF-kB activation, as well as activating ERK and JNK signaling cascades leading to STAT3 and MMP-2 stimulation, and BCL-2 expression. **(E)** Among the signaling pathways associated with the different phases of HCV-lymphomagenesis: IL-6 displays a pro-inflammatory effect, BLyS/BAFF is associated with HCV infection progression, and activates NF-kB, JNK and ERK pathways to stimulate B-cell proliferation, miR26b downregulation contributes to lymphomagenesis and NOTCH appears to deregulate DLBCL. **(F)** Cytokines IL-2, IL-6, IL-10, IL-12, sIL-2R, caspase 3/7 and BCL-2 are majorly

involved in HCV-Lymphomagenesis. Th1 cytokines: IFN-γ, TNF-α, and chemokines MIP-1α, MIP-1β, CXCR-3, CXCL10 and CXCL13, which display high expression levels, have potential implication in LPD pathogenesis.

Abbreviations. BCR, B cell receptors; MC, type II mixed cryoglobulinemia; TK, tyrosine kinase; LPDs, lymphoproliferative disorders; Igs, immunoglobulins; ER, endoplasmic-reticulum; ERK, extracellular regulated protein kinase; JNK, c-JUN N-terminal kinase; STAT3, signal transducer and activator of transcription 3; MMP-2, matrix metalloproteinase-2; DLBCL, diffuse large B-cell lymphomas; sIL-2R, soluble IL-2R.

a) HCV-induced chronic B-cell immune-stimulation leading to lymphoma

After the initial evidence for ongoing viral replication in lymphocytes from HCV chronic carriers, with or without MC and B-cell NHLs [11, 15, 16, 43], and with the involvement of BM [44-46], cumulative data correlated the presence of HCV in lymphoid districts and autoimmune LPDs in humans [12-14, 20, 72]. This was also confirmed by studies concerning both the persistence of HCV in *in vitro*-infected human lymphoid cells, injected into SCID mice [88], and of expression of the full-length HCV genome in B-cells from transgenic mice, spontaneously eliciting *in vivo* the B-cell lymphoma formation [89]. Due to its replication mechanism, HCV RNA may chronically stimulate the immune system, but other viral stimuli are also implicated, such as HCV surface antigens, which bind B cell receptors (BCR), and lymphocytic receptors expressed at the B cell surface [71, 72].

Persistent external stimulation in the B-cell compartment by HCV surface antigens binding BCR, tetraspanin CD81 and BCR, which plays an essential role in normal and malignant B-cells, may induce sustained polyclonal to oligo-monoclonal expansion of B-lymphocytes and overt B-cell lymphoma, mainly of the MZL-subtype (Figure 1) (Table 1) [70, 72]. A similar indirect mechanism has been implicated in *Helicobacter pylori*-related gastric mucosa-associated lymphoid tissue lymphoma (MALT), reviewed in [84, 85].

Table 1
Theories involved in the development of HCV-associated B-cell non-Hodgkin's lymphomas (NHL).

B-cell NHL subtype	Pathogenic mechanisms	Multistep processes involved in B-cell NHL pathogenesis				
		Oncogenic pathways		Factors/signals		
		Signaling	Genetic	Viral	Host	miRNA
DLBCL MZL SMZL	HCV-induced chronic B-cell immune-stimulation leading to lymphoma	CD81, CD80, CD86, CD21-R PD-1, SOCS-1 BCR, CD79A/B SYK, BTX, CARD11 IkBα, NF-kB BCL-2/BCL-xL, FAS	SHM NOTCH BCL-2	E2 NS3	IL-2, IL-6, IL-10, IL-17, TGF-	16, 155, 146a, 21, 26b, 125a, 125b, 138-5p, 147a, 147b, 511-rp, 139, 345, 126
FL	Chromosomal aberration in HCV-associated lymphomagenesis	BCL-2/BAX	t(14;18) BCL-2			
DLBCL MZL FL	Direct HCV-infection of B-lymphocytes produces permanent genetic damage	AID, TNF-α	BCL-6 TP53 CTNNB1	Core NS3		16, 155, 146a, 21, 26b, 125a, 125b, 138-5p, 147a, 147b, 511-rp

DLBCL MZL	Direct oncogenic role of HCV in B-lymphocytes	DNp63/73, P13K TNF-α, TNFR, TLR2 IL-6, IFN, CD95, NOS, ROS CHK2, HuR, ATM ERK, JNK, MMP-2 BAX, NaPB, NF-kB AP-1, SRE, STAT3	TP53 BCL-2 TP53	Core NS3/4A NS4B NS5A	IL-2, IL-6, IL-10 sIL-2Rα, IL-8	16, 155, 146a, 21, 26b, 125a, 125b, 138-5p, 147a, 147b, 511-rp
DLBCL MZL SMZL	Signaling pathways potentially involved in HCV-lymphomagenesis	RAS, RAF, MEK, IFN JAK, STAT ISGs, MVP, IFN-1, IL-6 BLyS, TUR2, INF, ERK Ostepontin, IL-1β	NOTCH SHM PTEN		IL-6, IL-2	16, 155, 146a, 21, 26b, 125a, 125b, 138-5p, 147a, 147b, 511-rp 139, 345, 126
DLBCL MC	B-cell lymphoproliferation mediated by cytokines and chemokines	IL-2R, IL-10 BLyS/BAFF CD19, sIL-2Rα IFN-γ, TNF-α CXCR-3, MIP-1α, MIP-1β	BCL-2	NS3 Core	IL-2, IL-10, sIL-2Rα Caspase 3/7	16, 155, 146a, 21, 26b, 125a, 125b, 138-5p, 147a, 147b, 511-rp

Abbreviations: DLBCL, diffuse large B-cell lymphomas; MZL, marginal zone lymphoma; SMZL, splenic marginal zone lymphoma; FL, follicular lymphoma; MC, type II mixed cryoglobulinemia; PD-1, programmed death-1; SOCS-1, suppressor of cytokine signaling; BCR, B cell receptors; SYK, spleen tyrosine kinase; BTK, Bruton's tyrosine kinase; SHM, somatic hyper-mutation; AID, activation-induced cytidine deaminase; NOS, nitric oxide synthase; ROS, reactive oxygen species; PI3K, phosphoinositide 3-kinase; TNFR, tumor necrosis factor receptor; TLR2, Toll-like receptor 2; CHK2, checkpoint kinase 2; HuR, human antigen R; ATM, ataxia telangiectasia mutated; ERK, extracellular regulated protein kinase; JNK, c-JUN N-terminal kinase; MMP-2, matrix metalloproteinase-2; NaPB, sodium phenylbutyrate; AP-1, activating protein-1; SRE, serum responsive element; STAT3, signal transducer and activator of transcription 3; ISGs, IFN-stimulated genes; MVP, major vault protein; TUR2, Turion 2; BLyS/BAFF, B-lymphocytes stimulator/B-activating factor; sIL-2R, soluble IL-2R

High-affinity binding between HCV-E2 and tetraspanin CD81, expressed at the surface of B-cells [70], enables formation of a B-cell costimulatory complex with CD19, CD21, and CD225 (the interferon-inducible Leu-13). This complex, together with BCR, lowers the threshold required for B-cell activation by joining antigen-specific and CD21 mediated complement recognition [90, 91].

Specifically, the double binding of HCV-E2 to the CD19/CD21/CD81 complex and BCR by specific HCV antigens, results in a reduced threshold for B-cell activation and proliferation [72], or alternatively the involvement of CD81 binding alone may be sufficient to induce robust B-cell proliferation, even in the absence of BCR co-ligation [71]. CD81 attachment to B-cells by combining the viral E2 protein with anti-CD81 mAbs, enabling proliferation of naïve B-cells and E2-CD81 binding, activates protein tyrosine phosphorylation and hyper-mutation of Ig genes in B-cell lines [71, 84].

BCR signaling is involved in the development of normal B-cells and is critical for B-cell NHL development, but the involvement of BCR-signaling pathways in B-cells from the HCV-associated LPDs is still not clearly established. A recent study demonstrated the expression of HCV proteins in B-cells following HCV infection and showed that HCV upregulates BCR signaling in the primary B-cell compartment. BCRs include membrane Ig molecules associated with CD79A/CD79B (Ig-α/Ig-β) heterodimers [92]. Their binding to surface Ig antigens induces B-cell aggregation, which elicits SRC-family kinases to phosphorylate CD79A/CD79B with subsequent tyrosine kinase (TK) SYK, triggering a signaling cascade of Bruton's TK (BTK) and CARD11, which is a major component of the "signalosome" complex required for transduction of BCR signaling.

A new role has been recently recognized for the HCV NS3/4A protein in BCR signaling regulation during HCV infection-which highlights a novel molecular mechanism involved in HCV-associated LPDs. HCV NS3/4A protein overexpression interferes with checkpoint kinase 2 (CHK2) activity altering post-transcriptional human antigen R (HuR) gene regulation, thus modulating a network of target mRNAs associated with B-cell LPDs [92]. Moreover, NS3/4A is involved in antivirus immune surveillance by proteolytic cleavage activity, it interacts with ataxia telangiectasia mutated (ATM), and impairs DNA repair in cells other than the lymphoid compartment, making HCV-infected cells more susceptible to mutations and increasing chromosome instability.

Binding of CD81 to B-cells may also strongly increase T-cell proliferation by lowering the threshold for interleukin-2 (IL-2) production in response to suboptimal stimulation and bystander activation of B-cells, see [84] for review. In addition, evidence indicates that the programmed death-1 (PD-1) and suppressor of cytokine signaling (SOCS-1) pathways modulate the T regulatory (T_R) cell response by specifically suppressing T-cell functions in establishing HCV-associated lymphoma [18, 93]. Consequently, the number of $CD4^+CD25^+/CD8^+CD25^+$ T_R cells increased, which normally suppress the effector/memory T-cell response, and are critical in pathogen clearance, and therefore play a pivotal role in inducing HCV chronic infection.

Chronic antigenic stimulation of B-cells is relevant in the pathogenesis of HCV-related B-cell NHL and MC as a) HCV proteins not only act at the B-cell surface binding HCV receptors [70, 72], but also at HCV-containing immune complexes to RF-expressing B-cells, such as HCV NS3 protein cross-reacting with the IgM+RF cryoprecipitate [94]; b) Remarkably, characterization of RF B-cells, in germinal centers (GC) from hepatic follicles producing RF, or from post-GC, highlights the role of B-cell-originating lymphomagenesis due to somatic hyper-mutation and proliferation in response to antigen stimulation [41, 84].

Antigen-driven stimulation has been shown to play a crucial role in HCV-related lymphoproliferation: it involves mutations in the V(H) and V(K) genes belonging to the B-cell clone that induces B-cell NHL in patients with MC producing an IgM homologous to a protein with RF specificity [41]. This is supported by the evidence that chronically HCV-infected and virus-associated B-cell NHL patients displayed an antibody response involving use of the same *Ig V(H)* gene. HCV seems to predispose to the selection of a restricted B-cell repertoire in response to chronic antigenic stimulation, as WA anti-idiotype Igs, usually found in IgM_K of MC, preferentially make use of VH subfamily specific *VH1-69* and *VH3-7* genes, combined with specific VK genes, *VK3-A27* and *VK3-20*, as reviewed in [42, 73].

The involvement of VH1-69 with similar complementary determining region-3 (CDR3) suggests non-random and biased finalized use of the VH segment in this kind of HCV-associated NH lymphoma [95]. This is supported by the following evidence: the VH1-69 Ig segment is expressed in a restricted repertoire in fetal liver lymphocytes and is involved in natural immunity. Usually, in the adult, a productive WH1-69 rearrangement is detected in a minority (< 2%) of normal B-lymphocytes, while in chronic lymphocytic leukemia patients it is present in approximately 20 %; but only present in a very small minority of HCV-related MC cases. Sequence analysis of the clonal variable Ig (Ig V) gene from MC and HCV-related B-cell NHL patients indicated a restricted expression of VH and VL genes (*VH1-69* and *VK3-A27*), suggesting involvement of a common antigenic epitope which, following its selection and expansion in a B-cell clone, can give rise to an overt lymphoma [96]. Previous studies have demonstrated that HCV-driven clonal expansion of B cells in MC, having a memory phenotype, leads to production of VH1-69 natural antibodies (Abs) and escape to control mechanisms [97]. In a fraction of investigated cases, these cells may replace the entire pool of circulating B cells, without varying their number. Thus, this may both subvert homeostasis of B-cells and provide genetic alterations causing additional growth and giving rise to an overt lymphoma.

The viral antigen(s) involved in B-cell clonal expansion have not yet been identified. In this regard, in monoclonal B-cell expansion of MC and in immunocytoma, the BCR in the monoclonal over-expanded B-cell subset, analogously to the IgM+RF cryoprecipitate, cross-reacts with the NS3 antigen of HCV [14, 94]. Another implication of HCV in lymphomagenesis has been provided by the binding of viral E2 envelope antigen to BCR cloned from an HCV-associated B-cell NHL. Therefore, in some HCV-associated B-cell NHLs and MC, it appears that B-cells respond directly to stimulation of viral antigens, since the Igs from one of the two tested HCV-associated lymphomas bound the E2 protein in a manner identical to human anti-E2 antibody [98].

Overall B-cell clonal expansion seems to be promoted by different HCV-related effects, including mutation/overexpression of B-cell proto-oncogenes, anti-apoptotic *BCL-2* by chromosomal translocation and interactions of HCV proteins with intracellular regulatory molecules, reviewed in [14], or with B-cell membrane receptors [70, 72], or binding of HCV-containing immune complexes to RF B- cells, as shown in mice [99].

There may also be an indirect role for HCV in LPD pathogenesis by means of host-immune response [100]. Indeed, in patients with chronic hepatitis C, B-cell functional activation was present in intrahepatic lymphoid follicles with GC formation, similar to those found in lymph nodes. HCV-associated immunocytoma can account for clonal proliferation of highly selected clones of B-cells that secrete cryoprecipitable RF, which is frequently encoded by the 51p1 VH gene combined with

the kv325 VL gene, thus confirming that an antigen-driven process supports a crucial role for clonal antigenic stimulation and evolution of B-cells in this lymphoma [101].

Treatment with E2-proteins or with viral particles produced from HCVcc, of cell line Raji, and of PBMCs, triggered IkBα phosphorylation; this led to overexpression of nuclear factor kappa B (NF-kB) and B-cell lymphoma-2 gene family proteins (BCL-2 and BCL-xL), and enhanced protection of both cell types from FAS-mediated cell death [102]. In addition, E2-CD81 signaling was accompanied by an increase in CD81 signaling and costimulatory CD80 and CD86, and a decreased in the complement receptor CD21.

Therefore, the E2-CD81 interaction may play a relevant role in HCV-induced LPDs and insufficient neutralizing immune response. Chronic antigenic stimulation plays a relevant role in initiating polyclonal expansion leading to genetic aberrations; however, the lack of ability of lymphoma BCR to bind HCV antigens in most of the investigated B-cell NHLs of patients with chronic viral infection supports the lack of generalization of this mechanism [103].

b) Chromosomal aberration in HCV-associated lymphomagenesis

Chromosomal translocations of immunoglobulin genes, which dysregulate oncogene expression placed under the control of Ig enhancers, are distinctive features of Ig gene remodeling in B-cell lymphomas [81]. Involvement of Ig loci in chromosomal translocation causes remodeling of Ig genes, during B-cell development, which generates antibody diversity by assembly of the V gene of the Ig heavy chain, through V(D)J recombination, somatic hyper-mutation (SHM) and isotype switching. Emblematic examples are the induced transcriptional dysregulation of B-cell lymphoma-1 gene *(BCL-1)* in mantle zonal lymphoma, *BCL-2* in follicular lymphoma (FL) and *MYC* in Burkitt's lymphoma, which are implicated in cell cycle, apoptosis inhibition and cellular growth regulation, respectively.

Chromosomal translocation 14:18 (t(14;18)) was also extensively observed in circulating B-lymphocytes from cases of chronic hepatitis C and MC [104-106], but not in liver biopsies from portal inflammatory infiltrates of HCV-infected patients [107]. This translocation is involved in malignant lymphoma, and is a transposition of the anti-apoptotic *BCL-2* gene, located in chromosome 18, joined to the 5' end of the IgH gene (IgH/*BCL-2*) on chromosome 14, causing overexpression of BCL-2 [108]. The most convincing evidence that HCV is directly involved in genetic changes observed in chronic viral infection and in benign HCV-associated LPDs, such as MC, is demonstrated by the disappearance of the t(14;18) translocation and Ig heavy chain (HC) gene rearrangement in most patients with chronic hepatitis after antiviral treatment [109]. This is reinforced by the reduced risk of developing lymphoma observed after obtaining a SVR following HCV treatment with IFN and RBV. Moreover, antiviral treatment is also able to abolish clonal B-cell expansion [18, 21, 85].

The t(14;18) translocation was firstly found associated with an increased frequency of the *BCL-2* gene rearrangement in cases of HCV-related MC [105, 109], with or without B-cell NHLs [110]; however this translocation was mainly found in follicular lymphomas, although in a low percentage (Table 1) [111]. The high-affinity binding of BCRs to HCV or to the envelope E2 protein [70, 71], can lead to proto-oncogene *BCL-2* activation by an increased frequency of t(14;18) [104, 105], compared to the frequency observed during B-cell development in uninfected immature B-cells of BM germinal centers to generate antibody diversity, reviewed in [81].

In HCV infected subjects the V(D)J rearrangement rate is amplified [104-106, 110], activating BCL-2 protein which is anti-apoptotic in B-cells due to a discrepancy of the BCL-2/BAX ratio, and enables survival of the anomalous B-cells. This is characteristic of the initial polyclonal expansion observed in MC, which represents an intermediate step before the development of frank B-cell NHL [104, 110].

B-cell expansion may stimulate production of autoantibodies, including the anti-IgG rheumatoid factor participating in IgG-M immune-complexes and MC [20]. Indeed, chronic antigenic stimulation plays an important role in the development of initial polyclonal B-cell expansion preliminary to genetic alterations, but a second hit, represented by altered expression of putative oncogene(s), can lead to overt lymphoma.

Remarkably, in these translocations t(14;18) the *BCL-2* gene is frequently found bound to the JH6 gene [112]. In particular, it has been observed that the major breakpoint region (MBR) of *BCL-2*, a 150-nt region of *BCL-2* exon 3 bound to a JH6 gene, was more often found in HCV-associated translocation t(14;18) compared to normal subjects. This suggests that, in the B-cell compartment of HCV patients, secondary D-to-JH rearrangements increase in frequency, and provide the molecular background for establishing most of the (14;18) translocations [112].

Since t(14;18) is eliminated after successful antiviral therapy [109], the reported anti-apoptotic effect of BCL-2 is implicated in progression to lymphomagenesis of HCV-infected B-cells, reviewed in [13, 14]. In chronic HCV-infection, aneuploidy increases with respect to the control [113]. Moreover, PBMCs from HCV patients and liver- derived hepatoma cell lines infected *in vitro* presented genetic instability associated with the low expression of retinoblastoma (Rb) protein, which is involved in cell cycle arrest when DNA abnormalities are detected. Mice that express the HCV Core protein also had low Rb expression, ruling out the notion that chromosomal aberration was only observed in tumor cell lines [114]. Cytogenetic analysis in primary splenocytes of these transgenic mice expressing Core protein showed at least twice the frequency of polyploidy compared to control mice. Lower Rb protein expression could avoid mitosis checkpoints contributing to cell polyploidy, thus promoting neoplastic transformation.

c) Direct HCV-infection of B-lymphocytes produces permanent genetic damage

Direct HCV-infection of B lymphocytes in chronic carriers may generate cellular transformation through hyper-mutations [14, 83-85], inducing permanent genetic damage and chromosomal alterations in B-cells.

Acute and chronic HCV replication has been shown to induce an enhanced mutation rate of immunoglobulin genes [115, 116] and proto-oncogenes [117] in B-lymphocytes, together with increased aneuploidy and polyploidy (Table 1) [113, 114]. The E2-CD81 interaction induces double strand DNA breaks and hyper-mutations of the heavy chain of Ig genes; in addition it lowers the affinity and specificity of HCV-specific antibodies, therefore enabling HCV to escape from immune surveillance [116]. A significant increase in mutation frequency found in IgHC, could also arise in *BCL-6, TP53* and β-catenin (*CTNNB1*) genes in *in vitro* HCV-infected B-cell lines, Raji, Ramos and the B-lymphoma derived JT [53], as well as in *in vivo* PBMCs, in lymphoma and HCC tissues derived from HCV-infected subjects [117].

However, contrasting evidence has been recently provided in this regard, demonstrating the absence of mutations in *CTNNB1* and *TP53* genes in naïve and memory B-cells from PBMCs of HCV chronic carriers, and in livers from HCV-infected patients [118]. Furthermore, both *CTNNB1*

and *TP53* mutated genes have been implicated in the development of HCV-associated HCC, bearing mutations in the target genes above reported [119, 120].

There was no significant difference in the mutation frequency for *BCL-6,* to which a SHM rate physiologically occurring in GC B-cells in the selected B-cell types infected *in vivo* by HCV is attributed, compared to B-cell subsets of healthy subjects [118]. These data reinforce a previous report, indicating, the absence of a significantly increased mutational rate in unselected PBMCs from chronically HCV-infected patients, with or without LPDs or lymphomas where, paradoxically a reduced mutational frequency was detected compared to healthy controls [121]. Further investigations are needed to evaluate to what extent the observed discrepancies depend on commitment, transformation state and modality of HCV infection of target lymphoid cells.

The reported permanent genetic B-cell damage, called "mutator B-cell phenotype", by the "hit-and-run" theory of cellular transformation is probably due to permanent genetic damages, caused by DNA double-strand breaks, inducing error-prone DNA polymerase, caused by activation-induced cytidine deaminase (AID), which also stimulates tumor necrosis factor α (TNF-α) production [115-117]. AID is overexpressed in B-cells of HCV infected individuals, suggesting that AID could play a crucial role in HCV-lymphomagenesis. In this regard, the CD19+ cell subset of B cells from PBMCs significantly overexpresses numerous genes correlated to lymphomagenesis, such as AID [122].

The high rate of chronicization of HCV-infection can be explained by molecular mimicry; this is an important immune-evasion strategy that provides host survival and persistence for several viruses. Structural and sequencing analysis has confirmed that the E2 protein of HCV displays neutralization epitopes in HVR1, and indicates that HCV mimics immunoglobulins; alterations in the mimicry domain are associated with the loss of ability to escape immune regulation [123]. Immunoglobulins, which are the effectors of human immunity, are tolerated "self" antigens; since the N-terminal domain of E2 has epitopes within HVR1 that are antigenically and structurally similar to the human Ig variable domain, E2 is a tolerated antigen directly involved in escape and persistence of HCV.

d) Direct oncogenic role of HCV in B-lymphocytes

HCV can cause HCC and is probably associated with papillary thyroid cancer [18]; therefore it can be considered an oncogenic virus [14, 73]. However, the mechanism of HCV-oncogenesis remains unclear; HCV neither replicates by reverse transcription nor integrates in the host genome or codes for known oncogenes [10, 92]. It is extremely unlikely that HCV could activate insertion mutagenesis or acquire cellular oncogenes by genetic recombination [67].

Mutations, deregulations and post-translational modifications in tumor suppressor genes, *RB1, TP53* and β-catenin/Wnt pathways [119, 120, 124] and/or other genes [10] were detected in HCV-associated HCC. However, the long latency of HCV infection has made it difficult to establish a causal relationship between HCV infection and proto-oncogene activation by mutations. HCV replication has been unequivocally demonstrated in hepatocytes from chronically infected subjects [40], thus supporting the hypothesis for direct involvement of HCV in HCC development. However, since not all HCV-associated HCCs have detectable HCV RNA [125] it has been postulated that a "hit-and-run" mechanism must be involved in HCV-carcinogenesis [117].

Transgenic mice expressing the full HCV genome in B cells were shown to spontaneously develop B-cell NHL *in vivo* [88]; however, evidence that HCV transforms human B lymphocytes *in*

vitro is still lacking [17]. It cannot be excluded that HCV exerts a direct oncogenic potential through some of its proteins that are known to be oncogenic, such as Core and NS3/4A, and other nonstructural proteins, e.g. NS4B and NS5A, see [10] for review.

Apart from being involved in basic functions of HCV life-cycle, all these viral proteins are also able to interact with signal transduction pathways involved in regulating cellular growth and proliferation in hepatic and extrahepatic districts; they display key activities for metabolic networks in apoptosis, transformation and IFN-signaling [126]. The presence of HCV has been reported not only in B-cell lymphoma, but in a number of healthy B-lymphocytes, such as monocytes $CD14^+/CD19^+$ [47] and in the CD27+ subclass, which is more resistant to apoptosis, and has therefore been indicated as a possible subset for an HCV reservoir in chronic hepatitis C [127]. Since persuasive evidence for HCV-permissiveness of T-lymphocyte has only been provided for the CD5+ subclass [87], it appears that the mechanisms commonly involved in HCV-lymphotropism, whose alterations could be relevant in lymphomagenesis, should differ considerably from HCV-hepatotropism, which is not mediated by CD5 expression.

The TP53 family isoform DNp63 was also found overexpressed in lymphoma [127] and is known to be overexpressed in other human cancers. In B-lymphoma cell lines expressing the HCV Core protein, and in primary B-lymphocytes from LPD patients, DNp63 was upregulated, while DNp73 was downregulated, providing an essential requirement for cell growth. In addition, phosphoinositide 3-kinase (PI3K) was required for Core dependent DNp63 transcription upregulation, strongly suggesting the involvement of deregulated PI3K in HCV-related LPDs [128].

Among the HCV proteins, HCV Core is the most pleiotropic protein, playing preeminent role in different signaling pathways concerning cell viability and proliferation [10]. HCV Core protein (the component of viral nucleo-capsid) is a multifunctional protein: it is involved in lymph node development, enhances TNF-α induced apoptosis, and promotes cell proliferation during HCV infection [129] owing to its ability to bind the cytoplasmic domain of the TNF-receptor [130]. Recently, very high levels of soluble TNF-receptors I and II were found associated with HCV-dependent MC and B-cell NHL, strongly suggesting that they were actively involved in LPDs [131].

The HCV Core protein can also induce IL-6 in $CD14^+$ cells via Toll-like receptor 2 (TLR2) leading to increased B cell proliferation [132]. In addition to hepatocarcinogenesis [133], Core protein is closely linked to lymphomagenesis in transgenic mice [134]. Indeed, RNA expression of HCV Core protein in enlarged lymph nodes, the disruption of IFN-signaling, associated with lymphoproliferative stimulus by type II CD95, caspases and interleukins, were strongly involved in development of LPDs and frank NH lymphoma [135]. Molecular profiling of human B lymphocytes expressing HCV Core provided evidence for significant changes in the expression of gene categories regarding cell death/apoptosis, hematological malignancy, antigen presenting and processing [136].

HCV infection is associated with B-cell NHLs, specifically MZL and DLBCL, while chronic antigenic stimulation is the main determinant in MZL, whose direct oncogenic role is supported by *in vitro* studies (Table 1). *In vitro* expression of HCV Core protein and NS3 proteins have been shown to induce nitric oxide synthase (NOS) and reactive oxygen species (ROS) generation; these cause DNA repair damage and mitochondrial injury that may precede cellular transformation [137].

A recent study conducted *in vivo*, by *in situ* NS3 immunostaining in biopsies from DLBCL and MZL patients, showed a strong association between NS3 protein detection and high-grade lymphomas [138]. NS3 has been previously reported to transform mammalian cells *in vitro*, see [10, 126] for review.NS5A protein disturbs mitogen signaling pathways, thus allowing protection from

TNF-α and TP53; NS5A induces apoptosis and promotes tumor growth and is a potential viral BCL-2 homologue that interacts with BAX. NS5A also inhibits sodium phenyl butyrate-induced apoptosis in Hep3B HCC cells [139]. HCV Core protein and NS5A behave as transcriptional trans-activators for a number of cellular activities such as NF-kB, activating protein-1 (AP-1), serum responsive element (SRE) and signal transducer and activator of transcription 3 (STAT3).

NS4B is known to modulate NS5A hyper-phosphorylation and trans-activate IL-8, reviewed in [126]. Moreover, it induces an unfolded protein response and endoplasmic-reticulum (ER) overload response-dependent NF-kB activation, activates the extracellular regulated protein kinase (ERK) and c-JUN N-terminal kinase (JNK) signaling cascades, leading to stimulation of STAT3 and matrix metalloproteinase-2 (MMP-2) activity and BCL-2 expression [140].

Significant stimulation of signal transduction and STAT3, MMP-2 and BCL-2 activation occurs in PBMCs from persistently HCV-infected patients and in cultured cells [140]. The mechanism by which HCV regulates STAT3, MMP-2 and BCL-2 has been recently studied. HCV allows regulation of MMP-2 and BCL-2 by activating the STAT3 signaling cascade; conversely STAT1/2 is upregulated by ERK, JNK and protein kinase C (PKC) pathways. In particular, JNK mediates extracellular signal transduction to the nucleus, activating cell proliferation/apoptosis and differentiation, as well as stress-mediated signaling by transcription factors [140].

e) Signaling pathways potentially involved in HCV-lymphomagenesis

Evolution of viruses means that they can modulate host-cell signaling pathways, favoring their replication. These modulations are a consequence of the binding of the virus to its host-cell receptor, cross-interactions between viral and cellular proteins and stress caused by infection. Several viruses are known to activate RAS/ RAF/ MEK metabolic pathways during early infection. Investigating the relationship between activation of these pathways and HCV replication has enabled the conclusion that HCV infection *per se* activates RAS, RAF and MEK pathways, which in turn stimulates HCV replication (Table 1) [141], thus providing an example of an evasion mechanism of the host antiviral system from HCV. This effect is mediated by attenuating the IFN-JAK-STAT pathway and is accompanied by downregulation of IFN-stimulated genes (ISGs), while HCV-activation of major vault protein (MVP) suppresses virus replication through IFN-1 expression [142].

HCV infection is associated with lethal diseases, such as HCC and B-cell NHL, causing a serious global health problem. However, the pathogenic mechanisms of these neoplasms remain largely unknown. HCV activates multiple cell signaling pathways; the signal transduction network dependent on the regulation of HCV infection, and the molecular machinery triggering this regulation have been recently investigated [10, 126]

IL-6 is involved in the development of MCs and B-cell NHLs in HCV-infected patients by TLR2 stimulation [85]. Expression of B-lymphocytes stimulator/B-activating factor (BLyS/BAFF), a cytokine belonging to the TNF-family, in splenocytes of transgenic mice, is associated with the clinical progression of HCV infection and may activate NF-kB, JNF and ERK pathways, stimulating B-cell proliferation. Investigations of the polymorphism of the *BAFF* gene promoter, such as the allelic variant -817, which correlates with an increased BAFF transcriptional activity [14, 143], have revealed a more frequent *BAFF* gene over-expression in sera from HCV-positive subjects with MC than in controls without MC [144, 145]. In addition, increased levels of osteopontin and IL-1β were observed in sera from patients with HCV-positive B-cell NHL. In HCV-positive subjects, the highest levels of osteopontin and IL-1β were closely associated with MC, regardless of B-cell lymphoma

development [146-148]. Expression profiles of lymphoma B-cells from HCV-transgenic mice displayed an altered expression of various genes [14, 84, 89, 126]. In particular microRNAs may act as effective biomarkers of DLBCL [149].

The NOTCH signaling pathway was deregulated in HCV-positive DLBCLs. Comparative analysis indicated that NOTCH2-mutations were found in 20% and NOTCH1-mutations in 4% of the HCV-cases compared to only 1/64 of HCV-negative ones [150]. PTEN mutations were only detected in 2% of cases. These mutations were associated with poor overall survival and histopathological alterations typical of DLBCL cell transformation. Since NOTCH1 is involved in the pathogenesis of splenic marginal zone lymphoma (SMZL), these data fit well with the reported observation that a high proportion of DLBCL in HCV-positive subjects can arise from a preceding MZL [18].

f) B-cell lymphoproliferation mediated by cytokines and chemokines

Numerous experimental evidence, conducted *in vivo* and *in vitro*, unequivocally supported that HCV replication takes place in B cells, reviewed in [17]. In these cells, direct involvement of HCV RNA (-) and the viral NS3 protein expression in HCV mRNA replication, translation and polyprotein production was demonstrated.

However, the principal HCV-target population of the immune system is still unknown. Major mechanisms involved in HCV-associated lymphomagenesis were found to be: induction of cytokines IL-2, IL-10 and the soluble IL-2R (sIL-2R), caspases 3/7 and BCL-2 in relation with the intracellular expression of oncogenic viral NS3 and Core proteins, as well the role played in activating IL-12 by HCV-E2 [14, 18] (Table 1). Although recent studies have strongly supported the oncogenic role of the HCV genome or some of its viral proteins in B lymphocytes [84, 85], the potential HCV penetration and replication in B lymphocytes still remain to be clarified. Penetration seems to be restricted to the CD5+ subset of the B-cell population, which also overexpresses CD81 [86]. It is also possible that for full permissiveness of B cells, other events, such as the EBV coinfection, may be required [83].

The immune response is modulated in particular by cytokines and chemokines, whose balance between activation and repression may induce several autoimmune diseases. Recent evidence has highlighted the role of cytokines and chemokines in the pathogenesis of HCV-related LPDs [14]. In both normal and leukemic B-lymphocytes IL-2R (Tac/CD25) expression is upregulated by IL-10 [151]. Consequently, both IL-2R and IL-10 may be involved in B-cell transformation of B-cell NHL, while BLyS/BAFF cooperate to induce the B-cell survival observed in LPDs.

Soluble interleukin receptor-α (sIL-2Rα), is involved in several types of cancers [152], including T-cell lymphomas [18]. In fact, sIL-2Rα overexpression in splenocytes correlated with a high incidence (25%) of DLBCL development in HCV-transgenic mice (RzCD19Cre), which express the full-length HCV-genome, under CD19-gene transcriptional control, in B-cells [89].

The role for cytokines and chemokines has been supported by recent studies, mainly in MC, where Th1 cytokines, such as IFN-γ and TNF-α and other chemokines (MIP-1α, MIP-1β, CXCR-3, CXCL10 and CXCL13) are highly expressed, as observed in vasculitis lesions, signifying a potential implication in pathogenesis [153]. CXCL13, which is required for B-cell trafficking, is another chemokine upregulated in MC [154], being overexpressed in liver biopsies from active cutaneous vasculitis. Since HCV-related MC patients with autoimmune thyroiditis present high serum levels of

CXCL10 and CXCL11, their role played in the MC pathogenesis should be investigated in depth [155].

THE IMPACT OF THE MICROENVIRONMENT AND miRNAs ON HCV-LYMPHOMAGENESIS

The role played in lymphocyte transformation via disruption of cell activity, uncontrolled cell division and immune function by environmental factors, such as inflammatory cells and soluble mediators leading to the development of indolent and aggressive B-cell lymphomas, has been the subject of extensive investigations in the field of lymphomagenesis and related viruses [156-158].

On one hand, it strengthens the causal correlation of HCV in many types of B-cell NHLs [18], on the other it raises the unsolved question of which other factors, e.g. in the environment, or those strictly involved in signal transduction pathways, impair basic cell functions in steps progressing along lymphoma development [158].

The interaction between lymphoma and the microenvironment helps explaining the geographical variability of HCV-induced B-cell NHLs [159]. It has been underlined that these imitate the regulated coexistence existing among malignant cells and the microenvironment, in analogy to the normal interaction between the latter and B-cells. The malignant development of the tumor cell growth requires external signals from the microenvironment namely the involvement of virus antigens, cell-cell interactions, cellular cytokines (IL-6, IL-7, IL-10, TGF-β), chemokines (CXCL1, 10, 11, 13) and caspases (Table 1) [18, 132, 147, 151, 155].

In this process, collaborating the lack of surveillance induced by viruses carrying immune deficiency, and immune stimulation dependent on lymphocyte activity by unknown or re-emergent factors, viruses are enclosed [158], possibly by involving a “hit-and-run” mechanism, as postulated for the HCV, susceptible to inducing hyper-mutations in Igs and proto-oncogenes [117].

Mutually non-exclusive pathogenic hypotheses were proposed to explain HCV-lymphomagenesis, after an initial "switch" of B-cell clonal proliferation by HCV antigenic stimulation, followed by chromosomal aberrations acting on the 14;18 translocation and *BCL-2* gene activation. Further costimulatory or successive "hits" should cooperate to drive infected B cells toward full malignancy. In addition to the described hyper-mutations in Igs and oncogenes/anti-oncogenes, which are under direct viral infection stimulus-by E2-CD81 binding at the cell surface, displaying transforming potential mediated by NOS, Core and mutagenic NS3/4 viral proteins, miRNA (or miR) dysregulation also plays a role (Table 1) [85, 149].

The role of miRNAs in gene silencing has recently been characterized in the eukaryotic gene regulation [159]. These miRs are highly conserved; they act as post-transcriptional regulatory elements that control gene expression by complemental base pairing at the 3’-UTR binding of target mRNA transcripts, enabling degradation or suppression of translation. Since a single miRNA targets multiple mRNAs, it is able to regulate the expression of many genes; miRNAs have a high tissue specific expression pattern and were found actively involved in organogenesis and carcinogenesis [160, 161], as well as viral pathogenesis and immunity [162].

Furthermore, several oncogenic DNA viruses code for miRs, thereby modulating the regulation of several cellular genes functional for their replication; nevertheless, this does not happen for HCV and other RNA viruses linked to infection and cancer. However, a new class of virus-

derived small RNAs (vsRNAs), showing very high homology to infectious viral genomes, has been described [158].

miRs modulate host-cell mRNA expression leading to important phenotypic changes of cell inflammation, differentiation and proliferation, favoring pathological processes leading to neoplastic transformation. One example is the hepatic specific miR-122, which regulates pathways that maintain liver-specific functions [161], and promotes HCV RNA replication [39]. Upregulation of miR-155 has been associated with NHLs and other solid and hematologic malignancies; the miR-17-92 cluster is specifically involved in B-cell lymphoma, while miRNA-143 and miR-145 expression are reduced [158]. It has been suggested that miRs are involved in activating both canonical and alternative NF-kB pathways [163], and down-regulation of miR-26b may contribute to lymphomagenesis. Reduced miR-26b expression has been demonstrated in HCV-positive patients with SMZL [85], in whom total subversion of the microRNA pattern was observed [164]. A significant decrease in miR-26b expression was also detected in PBMCs from MC and NHL patients, accompanied by an increase in miR-21, miR-16 and miR-155 expression, in NHL only [165]. The reduced expression of miR-26b accompanied the decrease in levels of miR-139, 345, 125a and 126 [164]. Overexpression of miRs 21, 155 and 146a was shown in splenic tumor tissues from SMZL patients, compared to normal controls. A decrease in miR-138-5p expression, together with a greater expression of miR-147a/miR-147b and miR-511-rp, is related to poor prognosis in HCV-positive DLBCL [149].

ANIMAL MODELS FOR INVESTIGATING HCV- LYMPHOMAGENESIS

In the last decade, transgenic mice have been successfully used to investigate HCV-lymphomagenesis [166]. Humans and primates are the only species susceptible to HCV infection and develop persistent viral infection. More recently, a genetically-humanized rodent model and xenotransplanted and hepatic chimeric mice were very promising for investigating the signaling pathways and molecular steps involved in this form of virus-associated lymphomagenesis [14, 18, 72, 78, 84, 114, 126].These alternative animal models are useful in investigating the characteristics of the HCV replication cycle and steps of viral infection.

HCV-lymphomas are heterogeneous [18, 73, 75]; different oncogenic pathways may be predominantly involved, according to the subtype [83-85, 167], and transgenic mice are therefore important to discover the mechanisms implicated in pathogenesis.

Viral replication in lymphocytes was demonstrated in patients with HCV-chronic infection with or without MC [11, 15-17, 43], and the involvement of BM was shown [44-46]. Cumulative data then correlated the presence of HCV in lymphoid districts with autoimmune LPDs in humans [12-14, 41, 42, 72]. This was highlighted in a study investigating HCV-persistence in *in vitro*-infected human lymphoid cells injected into SCID mice, which revealed the presence of HCV RNA in serial B-cells sub-passaged in these animals [88]. HCV RNA - it-self - may chronically stimulate the immune system to induce B-cell NHL in mice [89]; as well as other viruses, see [14] for review.

Transgenic mice expressing in their splenocytes BLyS/BAFF, a cytokine belonging to the TNF-family, had clinical progression of HCV infection. Moreover, several pathways stimulating B-cell proliferation were activated (NF-kB, JNK and ERK), reviewed by [85]. To exclude that chromosomal aberrations were only observed in cancer cell lines, mice expressing HCV Core protein were studied by cytogenetic analysis; their primary splenocytes displayed nearly twice the frequency

of polyploidy than the control mice [100]. Lower Rb protein expression could avoid mitosis checkpoints contributing to cell polyploidy, thus promoting neoplastic transformation. The HCV Core protein in transgenic mice was shown to induce HCC [133], and malignant lymphoma [134, 168]. The expression of HCV Core RNA in enlarged lymph nodes and the observed disruption of INF-signaling were strongly related to the development of LPDs and frank NH lymphoma.

Both IL-10 and IL-2R could be involved in B-cell transformation. Functional IL-2 receptor Tac/CD2 expression is upregulated by IL-10 in normal and leukemic B-lymphocytes [151]. Indeed, transgenic mice RzCD19Cre expressed high levels of sIL-2Rα in B-cell splenocytes and in 25% developed DLBCL. These mice contain the full-length HCV-genome under the B lineage-restricted CD19-gene transcriptional control [89, 168], strongly supporting the evidence that persistent HCV expression could be directly involved in B-cell lymphomagenesis. To investigate the signaling pathways of HCV-associated B-cell NHLs, comprehensive molecular analysis was performed in HCV transgenic mice RzCD19Cre using a genome-wide microarray [163]. Expression profiles of lymphoma B-cells from HCV-transgenic mice displayed an altered expression of various genes, such as *FOS*, *C3*, *LT R, A20,* miR-26b and *NF-kB*; this suggested that activation of both canonical and alternative NF-kB pathways and down-regulation of miR26b actively contribute to lymphomagenesis, since reduced expression of miR-26b has been found in HCV-positive SMLZ patients [164].

There was a correlation of tumor volume with the concentration of tumor-derived soluble IL-2R in body fluids of host SCID mice xenografted by human T-cell lymphoma [169]. Elevated sIL-2R serum levels in lymphoid malignancies correlate with the clinical stage of disease. A second murine model is the Irf1--/CN2, in which HCV-structural proteins (E1, E2, NS2 and Core) disrupted IFN regulatory factor-1. These mice have a high prevalence of lymphomas and LPDs, as well as increased levels of IL-2, IL-10 and BCL-2 together with BCL-2 expression. Therefore, in this mouse model, overexpression of apoptosis-related proteins and/or aberrant cytokine production was the primary event involved in inducing lymphoproliferation [168].

Functional proof that genes associated with leukemia/lymphoma were under HCV Core upregulation was reported in mice, in which the core gene was expressed by an adenoviral vector [136]; apoptosis was inhibited and there was a dramatic down-regulation of MHC class II in primary B-lymphocytes. Suppression of FAS-mediated cell death and apoptosis was observed in a transgenic mice model expressing Core, E1, E2, and NS-2 HCV-proteins under the control of the CD2 promoter [170]. In these mice, release of cytochrome C from mitochondria was abolished by expression of viral proteins that inhibited activation of caspases 9 and 3/7, but not caspase 8, thus indicating that these caspases are the targets of above viral proteins implicated in the suppression of FAS-mediated cell death and apoptosis.

HCV Core protein -mediated injury in transgenic mice liver is driven by reduced IFN γ and IL-2 production, similarly to that in chronic HCV-infected subjects, indicating interaction of FAS-mediated apoptosis in the liver infiltration of peripheral T-cells [171]. A similar mechanism of core expression in T-lymphocytes may play a crucial pathogenic role in immune dysregulation by liver T-lymphocyte infiltration and injury.

CONCLUSIONS

The mechanism(s) of HCV-lymphomagenesis remain, in part, elusive. Some pathogenic features of HCV-associated LPDs and B-cell NHLs have been clarified [18, 85]: 1) virus lymphotropism and 2) immune escape in the close association of HCV with MC and B-cell NHL [11-13, 17, 172, 173], and some of the major virus/host factors involved have been identified [157-159].

Direct and indirect mechanisms in HCV lymphomagenesis have been widely reviewed [14, 19, 20, 42, 72, 73, 78, 84, 127, 168, 174] namely anti-apoptotic mechanisms, and clonal expansion. The latter is mediated by viral-modulation of growth regulatory signals and oncogenic pathways, through the hyper-expression and mutation of oncogenes/immunoglobulins and virus/host-induced immune evasion [41, 71, 92-96, 98, 102, 110, 114-117, 135, 137, 138, 143, 149, 165]. These result in uncontrolled cellular growth, including full malignant transformation [83, 130, 131, 140, 141, 149, 150, 163, 165, 175]. The different oncogenic mechanisms implicated appear to be mutually non-exclusive [83]. Their order, and how they may interact and integrate reciprocally to produce a synergic oncogenic stimulus, sufficient to drive HCV-infected B-cells toward lymphomagenesis, has not yet been entirely clarified in most known B-cell NHL subtypes [18], (reviewed in [175]).

The "*primum movens*" is the HCV-antigenic chronic stimulation of B-cells [88, 89, 92, 103] due to HCV-E2 binding to the CD81 complex and/or HCV-specific antigens to BCR [70-72, 98]. This binding results in a reduced threshold for B-cell activation and proliferation [90, 91] leading to abnormal cell growth and polyclonal expansion, preferentially in the MZL subtype and occasionally in MZL-derived DLBCL [167]. The E2-CD81 interaction induces hyper-mutations in the Ig gene of HCV-infected B-cells [115]. The mutation rate of the VH gene in Ig of B cells producing antibodies may lower the affinity and specificity of HCV-Abs, enabling HCV to escape from host immune surveillance [116].

The progression from pre-neoplastic LPDs and indolent benign low-grade lymphomas toward overt malignant B-cell NHLs has been suggested to occur via chromosomal translocations t(14:18) [104-106, 110]. This event causes cell proliferation through *BCL-2* gene hyper-expression [85], especially in the FL subgroup, but also in carriers of other HCV-associated B-cell NHL subtypes and MC [167]. The evolution of HCV-unassociated FLs was probably driven by GC reentries of B-cells overexpressing BCL-2, as demonstrated in mice [176]. In this case, the non-cognate bystander effect, which occurs in GC in HCV-unassociated FL and consists of T-cell help, primarly based on CD40 and cytokines, has been postulated to arise outside GC, involving other different mediators [167].

The "hit-and-run" theory in HCV lymphomagenesis [115-117], involves genetic mutations in known anti-oncogenes induced in HCV-infected liver cells, and was hypothesized but not demonstrated for hepatocarcinogenesis [119, 120]. Thus, involvement of this pathogenic mechanism in HCV-associated B-cell NHLs requires additional *in vivo* confirmation [118, 121].

In cases of HCV-positive DLBCL, translocations of *MYC* and/or *BCL-6* were detected, together with altered protein expression (CD37, IgA-G-M, MYC, BCL-2) and mutations of TP53 in tumor tissues [177]. Even patients with occult HCV infection presented greater expression of lymphoma-associated genes such as *AID*, *BAL*, cyclin D1 and 2, serine-*STK15* and *Gal-3* indicating that these genes are upregulated after viral therapy, in particular if HCV is detectable in PBMCs [178].

All the described theories indicate a crucial role for dysregulation of the microRNA network, since miR-26b downregulation is involved in weakening tumor suppression [164, 167].

The therapeutic potential of antiviral IFN+RBV therapy in low-grade HCV-related B-cell lymphomas demonstrates the role of HCV in the disease [19, 21]. However, the same therapies were ineffective in most patients with more aggressive lymphomas, such as DLBCL, associated with HCV in chronically infected subjects [18, 78, 179]. Two groups of DLBCL have been identified; namely *de novo* DLBCL, which is homogenously composed of large B cells; the second group is characterized by small B-cell infiltration originating from MZL transformation into the more aggressive DLBCL [167]. Thus, also in DLBCL patients, the occurrence of an additional evolution from benign low-grade B-cell NHL toward malignancy was driven by successive mutations in oncogenes/anti-oncogenes and/or genetic/epigenetic changes due to environmental factors [157-159].

B-cell NHLs are of different pathogenic grades and present diverse molecular targets. The therapeutic response in individual cases can therefore differ. In any case, HCV predisposes to all the direct or indirect oncogenic potential to interact with host B cells to reach a pre-neoplastic lymphoproliferative state, prior to full malignant transformation. Permissiveness to infection and commitment of B cells to transformation require further considerations [167].

The genetic predisposition in the HCV-induced HCCs and B-lymphomas is supported by accumulating evidence. Single-nucleotide polymorphism (SNP) analysis in the HLA class II *HLA-DRB1/DQA1* and *NOTCH4* genes revealed a close association with benign and malignant LPDs related to HCV infection. A genetic variant of *TNFAIP3/A20* (the *rs2230926G* allele), was more frequently found associated with patients with RF activity independently from the histological lymphoma subtype. Therefore, a minor innate A20 dysfunction causing NF-kB activation may result in the escape of autoimmune B-cells during chronic stimulation [167].

The HLA/KIR profile was investigated in HCV-related disease progression; KIRDS2 and KIR3DL2 variants were found in both benign and malignant HCV-associated LPDs. HLA-Bw6/KIR3DL1 combination of KIR and HLA haplotypes displayed an increased risk of developing lymphoma compared to MC; the HLA-Bw4/KIR3DS1 was also related to an augmented risk of HCV-associated HCC [180]. HCV chronic carriers have different probabilities of progression to severe liver damage or into LPDs depending on the presence of haplotype rs12979860 IL28b TL2R-174, demonstrating that innate immunity plays a role in HCV disease progression [181].

The genetic and molecular signatures of the diverse forms of HCV-related B-cell NHLs were considered in order to understand their pathogenesis [83-85]. They combined specific signatures and expression of different sets of genes associated with BCR expression, coupled with BLyS signaling; the latter was associated with the control of B cell maturation and NF-kB transcription factors [182]. The high heterogeneity associated with DLBCL malignancy can also result from *de novo* transformation of other more benign B-cell NHLs [78].

Depending on different genetic profiles, DLBCL can be of two subtypes: one derived and developed through GC reaction and the other from distinct phases of B-cell differentiation [156].

Proteomic analysis of B-cell lymphoma [183] helped classifying the HCV related one, based on protein expression profiles [167].

Expression profiles of lymphoma B-cells in HCV-transgenic mice displayed an altered expression of various genes [182], linked to activation of both canonical and alternative NF-kB and other oncogenic pathways. Gene expression profiling of B cell malignancies unrelated to HCV also allowed characterization of two distinct molecular forms of DLBCL; the first one expressed genes

characteristic of the GC, the second expressed genes normally induced during activation of B-cells *in vitro* [184]. Both showed pathways of different differentiation stages, based on their gene expression pattern, partly reflecting their tumor differentiation and proliferation rates, which are predictive in the host of clinical responses and outcomes [183, 185, 186].

B-cell NHL associated with MC vasculitis in HCV carriers can be identified on the basis of the signature of sCD-27, sIL-2R and gamma-globulin C4 levels, with diagnostic and prognostic potentialities [187].

All these mutually non-contrasting mechanisms drive the progression toward malignancy through different signaling pathways and successive development steps [83-85], and cooperate in the pathogenesis of HCV-lymphomas together with environmental factors [157]. The wide biological heterogeneity of HCV chronic carriers makes it difficult to predict which patients will progress to malignancy [188]. A prognostic and predictive early evaluation of therapeutic responses and outcomes of these tumors is crucial to antitumor therapies [73].

The recent success of DAAs [7-9, 79] in curing HCV infection needs further in-depth and longer clinical evaluation for assessing their effect on eradicating the different subtypes of B-cell NHLs. Treatment for lymphomas, moreover, could be ameliorated by novel therapeutic options such as CAR-T cell therapies [189, 190]. The anti-lymphoma DNA vaccines, targeting anti-idiotypic immunoglobulins, are so far limited to mouse models [191-193] although human vaccine trials have been initiated [194].

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Heterogeneity and coexistence of oncogenic mechanisms involved in HCV-associated B-cell lymphomas

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ABSTRACT

The association of HCV-infection with B-lymphomas is supported by the regression of most indolent/low-grade lymphomas following anti-viral therapy. Studies on direct and indirect oncogenic mechanisms have elucidated the pathogenesis of HCV-associated B-lymphoma subtypes. These include B-lymphocyte proliferation and sustained clonal expansion by HCV-envelope protein stimulation of B-cell receptors, and prolonged HCV-infected B-cell growth by overexpression of an anti-apoptotic *BCL-2* oncogene caused by the increased frequency of t(14;18) chromosomal translocations in follicular lymphomas. HCV has been implicated in lymphomagenesis by a "hit-and-run" mechanism, inducing enhanced mutation rate in immunoglobulins and anti-oncogenes favoring immune escape, due to permanent genetic damage by double-strand DNA-breaks. More direct oncogenic mechanisms have been identified in cytokines and chemokines in relation to NS3 and Core expression, particularly in diffuse large B-cell lymphoma. By reviewing genetic alterations and disrupted signaling pathways, we intend to highlight how mutually non-contrasting mechanisms cooperate with environmental factors towards progression of HCV-lymphoma.

Keywords: non-Hodgkin B-lymphoma, lymphomagenesis, hepatitis C virus, pathogenesis, mixed cryoglobulinemia, oncogenic pathways, genetic damage, chromosomal aberration

Running title: B-cell lymphoma and HCV

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GC conceived the writing, all authors contributed to the drafting, completing, and approval of the paper.

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INTRODUCTION

The hepatitis C virus (HCV), a member of the *Hepacivirus genus* of the *Flaviviridae* family, is a major cause for acute and chronic hepatitis worldwide, thus representing a major global health challenge [1]. Roughly 75% of HCV-infected subjects do not resolve acute infection and develop chronic hepatitis, an insidious condition that, in addition to causing steatosis and possibly liver failure [2], might evolve through fibrosis/cirrhosis into hepatocellular carcinoma (HCC) [3].

A deficit in host immune surveillance concerning humoral and cellular immunity, characterized by reduced cytotoxic T-lymphocytes (CTL), was shown to impair CD4+/CD8+ T-cell proliferation capacity and effector cell function [4, 5] allowing immune escape for the HCV and persistent viremia in chronic hepatitis C [6, 7]. Currently, despite the lack of an effective vaccine, direct-acting antiviral drugs (DAAs), alone or in combination, are able to cause viral clearance in most individuals infected with the major HCV genotypes [8, 9].

HCV-related hepatocarcinogenesis had not been fully clarified. This was ascribed to the inadequacy of *in vitro* models of HCV-infection, investigated to understand how the main oncogenic pathways of proliferation, regeneration and apoptosis, are modulated by the HCV in the infected hepatocytes [10]. Failure to identify all genetic/epigenetic alterations in oncogenes/anti-oncogenes and key genes in liver differentiation and angiogenesis, delayed understanding the mechanisms that drive transition of the HCV-infected hepatocytes from inflammation to malignant transformation, *via* fibrosis/cirrhosis. HCV chronic infection may also progress through some lymphoproliferative and autoimmune diseases, characterized by extrahepatic immune manifestations, which present different grades of aggressivity [11-13]. They encompass type II mixed cryoglobulinemia (MC) and monoclonal gammopathy of undetermined significance (MGUS) up to B-cell non-Hodgkin's lymphoma (B-cell NHL), reviewed in [14].

Increasing evidence, both *in vivo* and *in vitro,* for HCV lymphotropism [15-17] supports a direct involvement of HCV in lymphoproliferative disorders (LPDs) [18]. The primary pathogenic role of HCV in B-cell NHL was highlighted by the high prevalence of HCV infection in sera and replication in circulating lymphocytes from chronic carriers with MC and low-grade B-cell NHLs [11], as well as by efficacy of anti-viral therapy in eradicating most B-cell NHLs by viral load suppression after sustained virologic response (SVR), and by lymphoma relapse preceded by recurrence of viral load [19-21]. Unlike HCV-hepatocarcinogenesis [10], the heterogeneity of a wide range of LPDs that arise in chronic hepatitis C, their long development time and the large number of dissimilar pathologies and associated clinical evidence [13, 14, 18, 20], have prevented the formulation of a single, coherent model in HCV-lymphomagenesis.

Here we undertook a comparative analysis of the most common steps and oncogenic patterns, which can present synergy in the development of B-cell NHLs, by reviewing clinical and experimental studies, which have allowed elucidation of the main molecular pathways and viral/host factors involved in HCV-lymphomagenesis.

CELLULAR AND MOLECULAR TARGETS INVOLVED IN HCV-REPLICATION IN THE HOST

The HCV genome, a positive-strand (+) RNA of about 9,600 nucleotides (nt) [22] is composed of a long open reading frame of 9,024 nt, coding for a single polyprotein precursor of 3,000 amino acids (aa), flanked at the 5' and 3' ends by two highly conserved untranslated regions

(UTRs), required for viral RNA translation and replication. The 5' UTR is a non-coding region of 341 nt, containing an internal ribosome entry site (IRES) for translation initiation of a single polyprotein, cleaved in 10 proteins [23] by cellular and viral proteases. In the 5' UTR, interaction sites with miR-122 are located, upstream of IRES, which act as positive regulatory factors on RNA replication, stimulating translation and enhancing viral RNA replication. Downstream of the 5' UTR, several cis-acting RNA elements (CREs) are also positioned that, together with two stem loops in the core region, stimulate HCV RNA translation. Core and E1-E2 envelope glycoproteins are the major structural proteins of viral particles, and function as ligands for cellular receptors. The other seven proteins are nonstructural (NS) proteins. NS2, acting as a cysteine protease, is required for the NS2-NS3 junction cleavage generated by E2 cleavage that, together with the p7 viroporin protein, is involved in virus assembly. The five others NS proteins participate in the replicase complex: NS3 contains serine protease activity in the N-terminal domain, activated by the NS4A cofactor, while in its C-terminal there are NTPase and helicase activities required for RNA replication; NS4B triggers membrane rearrangement associated with the viral replication complex [24]; highly phosphorylated NS5A is involved in RNA binding, replication and assembly of HCV; NS5B functioning as a RNA-dependent RNA polymerase (RdRp) is the HCV replicase.

At the 3' end of 3' UTR a regulatory region of 200-235 nt is located, including a short variable region, a poly-U/UC tract of about 80 nt and the X-tail; a conserved RNA region of 98 nt that, along with the enclosed minimum poly (U) tract of about 25 nt, is involved in RNA replication [23]. The synthesis of the minus (-) strand HCV RNA, functioning as a replication intermediate (RI), starts at the 3' non-coding region (NCR) end of (+) strand genomic RNA. This mode of HCV RNA replication with (-) strand RNA serving as a template for producing a large excess of (+) strand RNA has been reported as asymmetric and semiconservative in others Flavoviruses. A recent model to explain regulation of the HCV RNA translation/replication switch has been proposed based on viral RNA circularization through interactions between IRES motifs in the 5' UTR and stem-loop structure at the 3' end in the NS5B coding region [25]. Overall, HCV RNA replication appears as a multistep process coordinated by viral and cellular proteins, and requires extensive intracellular membrane remodeling for biogenesis of replication factory and also exploits components of the intracellular lipid transport system for producing infectious virions [23].

Since the discovery of HCV by cDNA cloning identification of the virus genome [22], HCV molecular studies using HCV-recombinant clones and chimeric constructs have been conducted in *in vitro* cell models allowing efficient HCV-infection, replication and very high production levels of different virus genotypes, which can be expanded to different host cells [23, 24, 26-29]. In the last two decades, this approach has enabled a dramatic breakthrough in HCV molecular biology, unveiling virus coding capacity, polyprotein processing, protein functions, encapsidation, assembly and molecular targets in HCV replication [29-31] for contributing to developing effective antiviral agents and vaccines [9, 27].

Adaptation of robust cell systems for *in vitro* HCV infection has allowed the investigation of the molecular and enzymatic mechanisms of HCV RNA replication and has helped in clarify the main stages of the HCV life-cycle [23-29]. This has enabled the major progress in the basic knowledge of HCV entry and some clinical implications of infection [31].

However, the experimental strategy based on synthetic models [26, 27], which combines cell systems and surrogate infectious viruses not found in nature [32], has not greatly helped in understanding HCV pathobiology [17]. One of the main characteristics of native HCV RNA has not

been considered, i.e. to be present *in circulum* in the form of viral variants, called quasi-species, involving more than seven major genotypes and many more subtypes. Viral quasi-species (SVP) are due to the extraordinary genetic variability of HCV, ascribed to the high virus mutation rate caused by the error-prone mechanism of RNA polymerase [27] and these variants contribute to enlarging the viral host-spectrum.

THE DOUBLE HEPATIC AND LYMPHOID TROPISM OF THE HCV

Since the discovery of HCV as the etiological agent responsible for non-A and non-B hepatitis [22], HCV has also been found closely associated with a number of benign and malignant lymphoproliferative and autoimmune regulated diseases, principally the MC and B-cell NHL, mostly in countries with high prevalence of viral infection, on the basis of clinical-epidemiological surveys [11-14, 17-20].

Confirmation of the dual HCV-pathogenesis was achieved in extrahepatic and hepatic infected tissues investigating for HCV-virions by electron microscopy (EM) or immune-EM, for the presence of (+) and (-) HCV RNA strands by *in situ* hybridization (ISH) or highly strand-specific RT-PCR, using rTrh DNA polymerase to avoid or reduce false positivity [33], and for the expression of viral proteins by immunohistochemistry (IHC) or immunofluorescence (IF) [17].

The efforts to obtain HCV productive infection *in vitro* were firstly achieved by using the serum derived to inoculate primary fetal and adult differentiated hepatocytes [17, 34-38] or infecting hepatoma cell line-derived hepatic cells by recombinant/mutated HCV-strains, such as HCVcc and HCVpp cell systems [26-28]. Hepatic progenitor cells from human embryos have been found to be permissive to HCV infection and persistently produced the virus, whereas pluripotent stem cells were not permissive, with permissiveness dependent on liver specific miR-122 expression and cell factors involved in HCV replication [39].

The initial difficulties faced for detecting strong and persistent HCV replication in *in vivo* infected livers [40] and *in vitro* inoculated liver cell systems, using semi-quantitative and quantitative RT-PCR, privileged the search for virus lymphotropism by localizing HCV in lymphoid cells of extrahepatic districts in HCV-infected subjects and attempting to propagate the virus in cells of hematopoietic origin [17, 27]. Hence, over the last two decades, the pathogenic role played by the hepatic and lymphoid tropism of HCV has been widely investigated, not only in the establishment of persistent infection, but also in the induction of chronic inflammation and immune stimulation that may lead to hepatocarcinogenesis and LPDs and B-cell NHLs in lymphoid districts.

LYMPHOPATHOGENESIS RELATED TO HCV-LYMPHOTROPISM

The polymorphism of clinical manifestations belonging to the so-called "HCV syndrome" has been recently described in detail with particular emphasis on specific morpho-histopathological features and extrahepatic tissue localizations in relation to the diverse clinical courses of chronic hepatitis C preceding the evolution toward MC and B-cell NHLs [13, 20].

Type II Mixed cryoglobulinemia is characterized by an excessive production of autoantibodies and immune complexes in the serum, including mixed cryoglobulins (a mixture of different immunoglobulins (Igs), that can precipitate, becoming insoluble at low temperatures [12, 20]), whose monoclonal component is often associated with IgM with rheumatoid factor (IgM-RF)

activity and HCV RNA. Mixed cryoglobulins result from HCV-host interactions, which are dependent on numerous cofactors for pathogenicity. MC is a process characterized by deposition of circulating cryoglobulins on small vessels and is also called cryoglobulinemic vasculitis. MC is associated with a potential risk of developing B-cell NHL in about 10% of MC patients [13, 14].

MC defines HCV-driven immune mediated and lymphoproliferative disorders, followed by poly-oligo clonal B-lymphocyte expansion able to evolve into frank malignant lymphoma. The presence of lymphoid follicles in liver, mostly in cases of MC, similar to those found in HCV infection, indicates that these are sites of expansion of clonal B-cell, which also secrete RF [14]. Antigen-driven stimulation in HCV-related LPDs was demonstrated by the presence in the same patient of mutations in Ig variable heavy V(H) and variable kappa light V(K) genes of B-cell clones in premalignant and malignant lymphoproliferative manifestations, therefore representing sequential phases of the same antigen-driven pathological process [41].

Longitudinal analysis of immunoglobulin chain (IgH) mutations and expression of Ig antigen receptors, displaying sufficient homology with an anti-HCV protein, strongly confirmed that MC and the subsequent B-cell NHL were both antigen-driven LPD processes closely dependent on HCV infection. Therefore, MC can be considered a systemic autoimmune condition, characterized by cutaneous and organic involvement, which may be associated with chronic HCV-infection [20]. The hepatic involvement in MC, less frequent in systemic vasculitis, strongly suggests a hepatotropic role for HCV in this process. In particular, analogously in B-cell NHLs, MC mimics some immune-mediated disorders and malignancies, while demonstrating a pathogenic role of chronic HCV-infection in various MC forms associated with extrahepatic manifestations [13, 20]. These include rheumatic diseases and endocrine disorders, involving the thyroid, development of diabetes and, associated with sexual dysfunctions, a form of skin porphyria and a severe renal involvement of unfavorable prognosis.

Undeniably, B-cell clonal expansion characterizing MC seems to be the product of dysregulation of the immune system rather than the result of a true hematological malignancy [42]; several viral and host factors are required for MC development.

Evidence for HCV-lymphotropism. HCV lymphotropism has been clearly demonstrated [16-18], and represents a major advance in understanding the pathogenesis of HCV-associated B-cell LPDs leading to B-cell NHL. HCV replication was firstly observed in peripheral blood mononuclear cells (PBMCs) from *in vivo* HCV-infected patients with chronic hepatitis [14, 16]; HCV replication was also observed in MC/B-cell NHL [11, 43], in patients coinfected by HCV and HIV, and in some forms of occult HCV-infection [17], as well as in B-/T lymphocytes, macrophages, Kupffer cells, bone-marrow (BM) cells and dendrocytes [17, 44-49]. Furthermore, a virus inoculum derived from HCV-infected sera and other *in vitro* sources, was able to infect lymphoid cell cultures [50-54]. However, few systems for long-term culture of HCV have been obtained [17] by de *novo* and *in vitro* HCV-infection of B and T cells [54-56] or culture of B-cell lines persistently producing the infectious virus, derived from an HCV-positive lymphoma and presenting enhanced apoptosis [53]. This has important implications in HCV pathogenesis, demonstrating a cell-free virus sub-passage from HCV-infected macrophages to B-cells or hepatocytes [56], and also direct cell-to-cell HCV transmission from persistently HCV-infected human BM-derived lymphoblastoid cells to recipient hepatoblastoma cells [57] and by exosomes [58].

The reported evidences emphasized the relevance of the lymphoid compartment in establishing and propagating HCV infection, see [17] for review. However, conspicuous and

continuous virus production was unsuccessful by inoculating *in vitro* human B and T cells with wild-type virus strains, or using the JFH1-strain of HCV, which binds to but fails to infect B-cells [59]. There are many clinical and experimental investigations, which strongly confirm the existence of lymphotropism determinants in HCV proteins.

The role of HCV quasi-species. Increasing evidence has shown that HCV circulates in the form of heterogeneous RNA quasi-species, caused by high error-prone HCV RNA synthesis [60]. This has enabled better understanding of the extraordinary host-adaptation and variability that characterize some HCV variants [17]; it could be hypothesized that RNA quasi-species allow the virus to evade clearance by T and B lymphocyte immunity, leading to persistent infection in most infected subjects [61] as in the case of viral variants in the hypervariable region 1 (HVR1) of E2-envelope glycoprotein, under selective immunological pressure. Specific circulating viral quasi-species were firstly found compartmentalized in B-cells and monocytes [62] indicating the lymphoid district as a unique "*virus reservoir*" of the host-infected organism. Mutations specifically located in the 5' UTR of the HCV hepatotropic strain H77 enhance virus replication when grown in T lymphoid cells of the MOLT-4 cell line [52]. Thus, the presence of strain-specific sequences in the 5' UTR or different sequence heterogeneities in the E1 and E2 coding regions, can give rise to altered lymphotropism relative to hepatotropic virus strains [62, 63]. A quasi-species compartmentalization, observed in PBMCs from transplant recipients, strongly suggests that lymphoid tissues may also hold variants more susceptible to infecting lymphoid cells [64]. Compelling data has enabled identification of genetic determinants, within E1/E2, related to the entry lymphotropism, which in HCV chronic-infected HCV patients may convert the virus from hepatotropic to lymphotropic [65]. Indeed, a co-receptor B7-2 (CD86) was identified specific for infection by lymphotropic HCV in an HCV-positive B-cell lymphoma; this receptor was shown to mediate HCV infection of memory B-cells, leading to inhibition of their function and enhancing their differentiation into IgM-secreting plasmablasts [66]. Silencing of HCV replication in B-cells is promoted by virus sensor retinoic acid-inducible gene I (RIG-I) or microRNA-122 overexpression. Despite its low rate, the genetic recombination in HCV may also contribute to the observed virus genetic variations and display some pathogenic and clinical implications by generating escape mutants that confer drug resistance [67].

The involvement of HCV-cell entry. Before undergoing clathrin-mediated endocytosis and membrane fusion in the host-cell endosome, HCV enters the cells by interaction of the viral particle with the host-cell membrane. This involves prior attachment of E1 and E2 viral envelope glycoproteins with several cell cofactors, through a process involving extremely adjusted steps [31]. The HCV entry process requires glycosaminoglycans (GAGs) and low density lipoprotein receptors (LDL-R) for virus adhesion to the cell surface; then the tetraspanin CD81 (the first to be identified among the principal cell receptors/co-receptors), the high density lipoprotein receptor SR-B1, the two tight junction (TJ) proteins, claudin-1(CLND1) and occludin (OCLN), the Niemann-Pick C1-like 1 cholesterol adsorption receptor and the transferrin receptor 1 (TfR1) [68, 69]. Of these co-receptors, which are associated with the E1 and E2 HCV envelope proteins, CD81 binds the virus with a high affinity [70, 71], and appears to be the most ubiquitous, since it is overexpressed in B lymphocytes, as well as in hepatocytes and a number of different cells. This means that persistent stimulation of lymphocytes by viral surface antigens may be primarily implicated in the development of B-cell LPDs, such as MC and benign and malignant B-cell NHLs, see [14] for review. In fact, continuous antigenic stimulation of the HCV-induced B cell immunological response may lead to an initial oligo-polyclonal B-cell expansion, causing further genetic aberrations and mutations [71-73].

To explore the causal relationship between HCV infection and B-cell NHLs, environmental and genetic/epigenetic alterations, occurring in oncogenes and anti-oncogenes by only partially known mechanisms, have been investigated in-depth [14, 18, 20]. The subsequent clonal expansion of B cells following HCV infection may induce genetic modifications and alterations of signaling pathways involved in cell growth and survival leading to lymphoid cell transformation, which characterizes most of the current models in HCV-lymphomagenesis.

HCV INVOLVEMENT IN LYMPHOMAGENESIS: MORE THAN A SIMPLE SUSPICION

A number of epidemiological studies, recently reviewed [18, 19, 73], have supported the notion that HCV infection is a risk factor for B-cell NHL, playing a primary role in the development of premalignant and malignant lymphoproliferative diseases.

From meta-analysis of case-control clinical-epidemiological studies, the relative risk (RR), globally, of being infected by HCV was shown not to exceed a factor of 2.4 among patients with B-cell lymphoma relative to the general population [73]; HCV prevalence in B-cell NHL patients was about 15% respect to the controls [19]. The fraction of B-cell NHLs secondary to HCV-infection can reach 10-15% or even more in areas of high prevalence of HCV infection, like Italy and Japan, but decreases significantly in areas of low prevalence [18, 73]. About 5-10 % of benign MC progresses to lymphoma, while in HCV-infected patients with MC, the risk of developing B-cell NHL increases by 35-fold [74].

Most epidemiological studies addressed at establishing a causal relationship between chronic HCV-infection and B-cell lymphomas have mostly been inconclusive, especially when conducted in areas with low HCV prevalence, as well as studies that attempted to associate lymphoma risk with its histological subtype and HCV genotype [18, 19]. Antiviral treatment of HCV with interferon (INF) and ribavirin (RBV), demonstrated the regression of splenic lymphoma with villous lymphocytes [21]; this initial result was followed by numerous clinical trials that were successful in eradicating most indolent low-grade B-cell NHLs and MC by curing HCV, thus indicating a close causal link with HCV [19, 75].

A recent study enrolled a very wide homogeneous population (HCV cohort of more than 10,000 carriers and a non-HCV population of more than 40,000) and was followed-up for 8 years to minimize the inadequate temporal length, which in most of the studies reported may have considerably affected the NHL risk prevalence [76]; this study observed that the incidence rate of any type of lymphoid neoplasms, including B-cell NHLs (75%), was significantly greater ($P < 0.0001$) in the HCV cohort than the non-HCV cohort, 48.4 versus 22.1 per 100,000 person-years.

The three main histological subtypes frequently associated with HCV infection are diffuse large B-cell lymphomas (DLBCL), marginal zone lymphomas (MZL) and lymphoplasmacytic lymphomas (LPL) [77]. In a European multicenter case-control study (EPILYMPH), patients displaying HCV RNA positivity where associated with a greater risk of DLBCL. DLBLC, the most widespread large B-cell NH lymphoma in western countries, is also the most aggressive lymphoma, and there is a significant association between HCV infection and B-cell NHL. When associated with HCV, DLBCL displays an aggressive clinical form, which requires combined immune-chemotherapy with rituximab (anti-CD20) plus cyclophosphamide, vincristine, prednisone and doxorubicin (CHOP-R) [18, 73, 78]. Conversely, for HCV-associated indolent B-cell NHLs, the traditional

antiviral therapy (INF + RBV) was effective to achieve HCV eradication, and was followed by lymphoma regression in the majority of cases, reaching 75% in MZL [18].

IFN-free DAAs, as a first-line treatment for HCV, are effective in curing HCV in around 98% of chronic carriers harboring indolent B-cell NHLs [79]. The overall hematological response rate was 67% of cases (26% presented complete lymphoma remission). Of the MZL patients, the highest rate of lymphoma response was 73%. Therefore, particularly for patients with HCV-associated indolent lymphoma of this subtype, DAAs are a very promising safe antiviral treatment, while, for complete remission of patients with aggressive lymphoma, the DAA treatment required additional chemotherapy to be effective [80].

PATHOGENESIS OF HCV-LYMPHOMA: WHICH MECHANISMS ARE INVOLVED?

Causal agents of B-cell NHLs can be genetic factors [81] or acquired factors, namely environmental and infectious agents such as lymphotropic viruses: Epstein-Barr virus (EBV), human immunodeficiency virus (HIV), HTLV, herpes virus 8 and more recently also the HCV and the bacterium *Helicobacter pylori*, reviewed in [18, 73]. Among the risk factors that can induce host immunodeficiency favoring lymphoma development, reproductive factors such as hormones, have been recognized [82]. Other unidentified host-factors also play a key role, as suggested by the heterogeneity of histology, localization and clinical course of lymphomas related to infectious agents, or the discrepancy between the high prevalence of (often ubiquitous) infectious agents such as the EBV in B-cells of the infected carriers relative to the low percentage of patients developing lymphoma. In particular, it was recently hypothesized that each of the individual etiological agents that provide low oncogenic signals, are not strong enough to cause lymphoma alone, but may cooperate to produce a composite stimulus sufficient to give rise to a lymphoma [83].

Epidemiologists have suggested that developing B-cell NHL is a multi-step and multi-causal event, due to different etiologic mechanisms that can be mutually non-exclusive [83]. In fact, HCV-induced lymphomagenesis may also result from various oncogenic mechanisms [72, 81], acting through multi-stage and multi-factorial processes that may cooperate to develop a lymphoma [20, 84]. Experimental data [11, 53], has provided strong evidence for an oncogenic role of the HCV genome and/or of some of its viral proteins in infected B lymphocytes, reviewed in [85]. However, the role of virus interaction, penetration and replication in B-lymphocytes still remains to be fully clarified; the observed association of HCV with increased autoantibodies in MC and B-cell NHLs, and increased peripheral innate CD5+ B cells have suggested a pathogenic role for B cells in viral specific activation and clonal proliferation characterizing HCV-infection [84, 86]. Furthermore, convincing evidence has recently supported the fact that CD5+ cells only mediate HCV-infection in T-lymphocytes, without developing lymphoma [87].

Multistep processes allowing development of current models on lymphomagenesis associated with HCV, as outlined in Figure 1, include: a) HCV-induced chronic B-cell immune-stimulation leading to lymphoma; b) chromosomal aberration in HCV-associated lymphomagenesis; c) a direct HCV-infection of B-lymphocytes produces permanent genetic damage; d) direct oncogenic role of HCV in B-lymphocytes; e) signaling pathways potentially involved in HCV-lymphomagenesis; f) B-cell lymphoproliferation mediated by cytokines and chemokines.

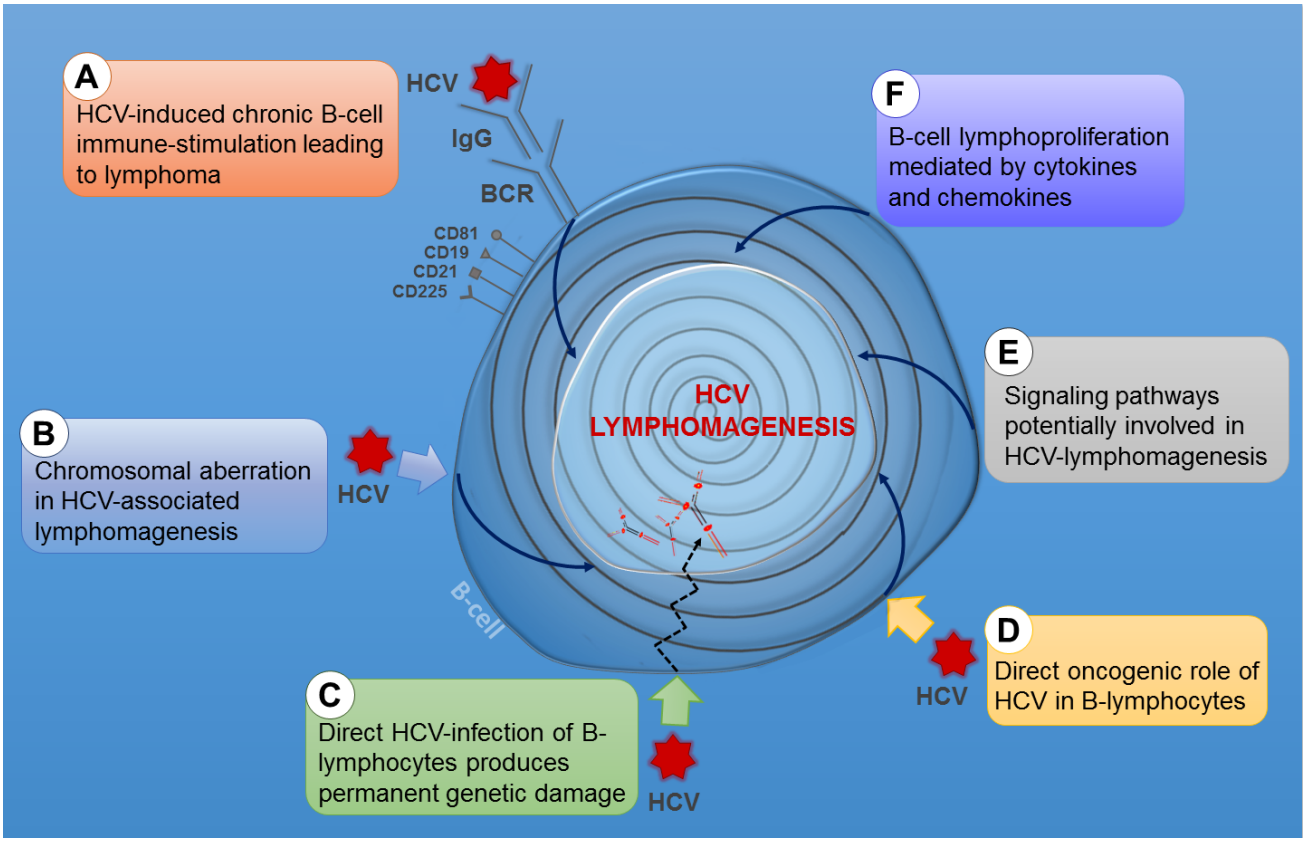

Figure 1. Current models of HCV-associated lymphomagenesis. (A) External antigenic stimulation of HCV-surface antigen binding CD81 and BCR receptors by HCV-envelope proteins induces proliferation and sustained clonal expansion of B-lymphocytes leading to MC and frank B-cell NHL. E2-CD81 binding enables the B-cell costimulatory complex by TK phosphorylation and Ig gene hyper-mutation. E2-CD81 signaling induces immune escape and LPD by an increase in costimulatory CD80/CD86 and a decrease in the complement receptor CD21. **(B)** Overexpression of anti-apoptotic *BCL-2* oncogene by increased frequency of chromosomal t(14;18) translocation prolongs HCV-infected B-cell growth preventing apoptosis, due to alteration of the BCL-2/BAX ratio. **(C)** HCV has been implicated in lymphomagenesis by a "hit-and-run" mechanism, inducing hyper-mutations of Igs in infected B-cells, and oncogenes/anti-oncogenes through permanent genetic damage, caused by DNA double-strand breaks inducing error-prone DNA polymerase by activation-induced cytidine deaminase that stimulates TNF-α production. **(D)** More direct oncogenic mechanisms have been explored, such as the role of the intracellular viral proteins as Core and NS proteins. HCV Core protein is required for cell growth and proliferation, upregulating TP53 family isoform protein DNp63 transcription and enhancing TNF-α induced apoptosis. NS3 + Core induces *in vitro* NOS and ROS, causing DNA repair damage and is closely associated with high-grade lymphomas *in vivo*. NS5A protects from TNF-α and TP53-induced apoptosis and promotes tumor growth. Core and NS5A behave as transcriptional trans-activators for several genes. NS4B modulates NS5A hyperphosphorylation and transactivates IL-8, inducing an unfolded protein response and ER overload response-dependent NF-kB activation, as well as activating ERK and JNK signaling cascades leading to STAT3 and MMP-2 stimulation, and BCL-2 expression. **(E)** Among the signaling pathways associated with the different phases of HCV-lymphomagenesis: IL-6 displays a pro-inflammatory effect, BLyS/BAFF is associated with HCV infection progression, and activates NF-kB, JNK and ERK pathways to stimulate B-cell proliferation, miR26b downregulation contributes to lymphomagenesis and NOTCH appears to deregulate DLBCL. **(F)** Cytokines IL-2, IL-6, IL-10, IL-12, sIL-2R, caspase 3/7 and BCL-2 are majorly

involved in HCV-Lymphomagenesis. Th1 cytokines: IFN-γ, TNF-α, and chemokines MIP-1α, MIP-1β, CXCR-3, CXCL10 and CXCL13, which display high expression levels, have potential implication in LPD pathogenesis.

Abbreviations. BCR, B cell receptors; MC, type II mixed cryoglobulinemia; TK, tyrosine kinase; LPDs, lymphoproliferative disorders; Igs, immunoglobulins; ER, endoplasmic-reticulum; ERK, extracellular regulated protein kinase; JNK, c-JUN N-terminal kinase; STAT3, signal transducer and activator of transcription 3; MMP-2, matrix metalloproteinase-2; DLBCL, diffuse large B-cell lymphomas; sIL-2R, soluble IL-2R.

a) HCV-induced chronic B-cell immune-stimulation leading to lymphoma

After the initial evidence for ongoing viral replication in lymphocytes from HCV chronic carriers, with or without MC and B-cell NHLs [11, 15, 16, 43], and with the involvement of BM [44-46], cumulative data correlated the presence of HCV in lymphoid districts and autoimmune LPDs in humans [12-14, 20, 72]. This was also confirmed by studies concerning both the persistence of HCV in *in vitro*-infected human lymphoid cells, injected into SCID mice [88], and of expression of the full-length HCV genome in B-cells from transgenic mice, spontaneously eliciting *in vivo* the B-cell lymphoma formation [89]. Due to its replication mechanism, HCV RNA may chronically stimulate the immune system, but other viral stimuli are also implicated, such as HCV surface antigens, which bind B cell receptors (BCR), and lymphocytic receptors expressed at the B cell surface [71, 72].

Persistent external stimulation in the B-cell compartment by HCV surface antigens binding BCR, tetraspanin CD81 and BCR, which plays an essential role in normal and malignant B-cells, may induce sustained polyclonal to oligo-monoclonal expansion of B-lymphocytes and overt B-cell lymphoma, mainly of the MZL-subtype (Figure 1) (Table 1) [70, 72]. A similar indirect mechanism has been implicated in *Helicobacter pylori*-related gastric mucosa-associated lymphoid tissue lymphoma (MALT), reviewed in [84, 85].

Table 1
Theories involved in the development of HCV-associated B-cell non-Hodgkin's lymphomas (NHL).

B-cell NHL subtype	Pathogenic mechanisms	Multistep processes involved in B-cell NHL pathogenesis				
		Oncogenic pathways		Factors/signals		
		Signaling	Genetic	Viral	Host	miRNA
DLBCL MZL SMZL	HCV-induced chronic B-cell immune-stimulation leading to lymphoma	CD81, CD80, CD86, CD21-R PD-1, SOCS-1 BCR, CD79A/B SYK, BTX, CARD11 IkBα, NF-kB BCL-2/BCL-xL, FAS	SHM NOTCH BCL-2	E2 NS3	IL-2, IL-6, IL-10, IL-17, TGF-	16, 155, 146a, 21, 26b, 125a, 125b, 138-5p, 147a, 147b, 511-rp, 139, 345, 126
FL	Chromosomal aberration in HCV-associated lymphomagenesis	BCL-2/BAX	t(14;18) BCL-2			
DLBCL MZL FL	Direct HCV-infection of B-lymphocytes produces permanent genetic damage	AID, TNF-α	BCL-6 TP53 CTNNB1	Core NS3		16, 155, 146a, 21, 26b, 125a, 125b, 138-5p, 147a, 147b, 511-rp

DLBCL MZL	Direct oncogenic role of HCV in B-lymphocytes	DNp63/73, P13K TNF-α, TNFR, TLR2 IL-6, IFN, CD95, NOS, ROS CHK2, HuR, ATM ERK, JNK, MMP-2 BAX, NaPB, NF-kB AP-1, SRE, STAT3	TP53 BCL-2 TP53	Core NS3/4A NS4B NS5A	IL-2, IL-6, IL-10 sIL-2Rα, IL-8	16, 155, 146a, 21, 26b, 125a, 125b, 138-5p, 147a, 147b, 511-rp
DLBCL MZL SMZL	Signaling pathways potentially involved in HCV-lymphomagenesis	RAS, RAF, MEK, IFN JAK, STAT ISGs, MVP, IFN-1, IL-6 BLyS, TUR2, INF, ERK Ostepontin, IL-1β	NOTCH SHM PTEN		IL-6, IL-2	16, 155, 146a, 21, 26b, 125a, 125b, 138-5p, 147a, 147b, 511-rp 139, 345, 126
DLBCL MC	B-cell lymphoproliferation mediated by cytokines and chemokines	IL-2R, IL-10 BLyS/BAFF CD19, sIL-2Rα IFN-γ, TNF-α CXCR-3, MIP-1α, MIP-1β	BCL-2	NS3 Core	IL-2, IL-10, sIL-2Rα Caspase 3/7	16, 155, 146a, 21, 26b, 125a, 125b, 138-5p, 147a, 147b, 511-rp

Abbreviations: DLBCL, diffuse large B-cell lymphomas; MZL, marginal zone lymphoma; SMZL, splenic marginal zone lymphoma; FL, follicular lymphoma; MC, type II mixed cryoglobulinemia; PD-1, programmed death-1; SOCS-1, suppressor of cytokine signaling; BCR, B cell receptors; SYK, spleen tyrosine kinase; BTK, Bruton's tyrosine kinase; SHM, somatic hyper-mutation; AID, activation-induced cytidine deaminase; NOS, nitric oxide synthase; ROS, reactive oxygen species; PI3K, phosphoinositide 3-kinase; TNFR, tumor necrosis factor receptor; TLR2, Toll-like receptor 2; CHK2, checkpoint kinase 2; HuR, human antigen R; ATM, ataxia telangiectasia mutated; ERK, extracellular regulated protein kinase; JNK, c-JUN N-terminal kinase; MMP-2, matrix metalloproteinase-2; NaPB, sodium phenylbutyrate; AP-1, activating protein-1; SRE, serum responsive element; STAT3, signal transducer and activator of transcription 3; ISGs, IFN-stimulated genes; MVP, major vault protein; TUR2, Turion 2; BLyS/BAFF, B-lymphocytes stimulator/B-activating factor; sIL-2R, soluble IL-2R

High-affinity binding between HCV-E2 and tetraspanin CD81, expressed at the surface of B-cells [70], enables formation of a B-cell costimulatory complex with CD19, CD21, and CD225 (the interferon-inducible Leu-13). This complex, together with BCR, lowers the threshold required for B-cell activation by joining antigen-specific and CD21 mediated complement recognition [90, 91].

Specifically, the double binding of HCV-E2 to the CD19/CD21/CD81 complex and BCR by specific HCV antigens, results in a reduced threshold for B-cell activation and proliferation [72], or alternatively the involvement of CD81 binding alone may be sufficient to induce robust B-cell proliferation, even in the absence of BCR co-ligation [71]. CD81 attachment to B-cells by combining the viral E2 protein with anti-CD81 mAbs, enabling proliferation of naïve B-cells and E2-CD81 binding, activates protein tyrosine phosphorylation and hyper-mutation of Ig genes in B-cell lines [71, 84].

BCR signaling is involved in the development of normal B-cells and is critical for B-cell NHL development, but the involvement of BCR-signaling pathways in B-cells from the HCV-associated LPDs is still not clearly established. A recent study demonstrated the expression of HCV proteins in B-cells following HCV infection and showed that HCV upregulates BCR signaling in the primary B-cell compartment. BCRs include membrane Ig molecules associated with CD79A/CD79B (Ig-α/Ig-β) heterodimers [92]. Their binding to surface Ig antigens induces B-cell aggregation, which elicits SRC-family kinases to phosphorylate CD79A/CD79B with subsequent tyrosine kinase (TK) SYK, triggering a signaling cascade of Bruton's TK (BTK) and CARD11, which is a major component of the "signalosome" complex required for transduction of BCR signaling.

A new role has been recently recognized for the HCV NS3/4A protein in BCR signaling regulation during HCV infection–which highlights a novel molecular mechanism involved in HCV-associated LPDs. HCV NS3/4A protein overexpression interferes with checkpoint kinase 2 (CHK2) activity altering post-transcriptional human antigen R (HuR) gene regulation, thus modulating a network of target mRNAs associated with B-cell LPDs [92]. Moreover, NS3/4A is involved in antivirus immune surveillance by proteolytic cleavage activity, it interacts with ataxia telangiectasia mutated (ATM), and impairs DNA repair in cells other than the lymphoid compartment, making HCV-infected cells more susceptible to mutations and increasing chromosome instability.

Binding of CD81 to B-cells may also strongly increase T-cell proliferation by lowering the threshold for interleukin-2 (IL-2) production in response to suboptimal stimulation and bystander activation of B-cells, see [84] for review. In addition, evidence indicates that the programmed death-1 (PD-1) and suppressor of cytokine signaling (SOCS-1) pathways modulate the T regulatory (T_R) cell response by specifically suppressing T-cell functions in establishing HCV-associated lymphoma [18, 93]. Consequently, the number of $CD4^+CD25^+/CD8^+CD25^+$ T_R cells increased, which normally suppress the effector/memory T-cell response, and are critical in pathogen clearance, and therefore play a pivotal role in inducing HCV chronic infection.

Chronic antigenic stimulation of B-cells is relevant in the pathogenesis of HCV-related B-cell NHL and MC as a) HCV proteins not only act at the B-cell surface binding HCV receptors [70, 72], but also at HCV-containing immune complexes to RF-expressing B-cells, such as HCV NS3 protein cross-reacting with the IgM+RF cryoprecipitate [94]; b) Remarkably, characterization of RF B-cells, in germinal centers (GC) from hepatic follicles producing RF, or from post-GC, highlights the role of B-cell-originating lymphomagenesis due to somatic hyper-mutation and proliferation in response to antigen stimulation [41, 84].

Antigen-driven stimulation has been shown to play a crucial role in HCV-related lymphoproliferation: it involves mutations in the V(H) and V(K) genes belonging to the B-cell clone that induces B-cell NHL in patients with MC producing an IgM homologous to a protein with RF specificity [41]. This is supported by the evidence that chronically HCV-infected and virus-associated B-cell NHL patients displayed an antibody response involving use of the same *Ig V(H)* gene. HCV seems to predispose to the selection of a restricted B-cell repertoire in response to chronic antigenic stimulation, as WA anti-idiotype Igs, usually found in IgM_K of MC, preferentially make use of VH subfamily specific *VH1-69* and *VH3-7* genes, combined with specific VK genes, *VK3-A27* and *VK3-20*, as reviewed in [42, 73].

The involvement of VH1-69 with similar complementary determining region-3 (CDR3) suggests non-random and biased finalized use of the VH segment in this kind of HCV-associated NH lymphoma [95]. This is supported by the following evidence: the VH1-69 Ig segment is expressed in a restricted repertoire in fetal liver lymphocytes and is involved in natural immunity. Usually, in the adult, a productive WH1-69 rearrangement is detected in a minority (< 2%) of normal B-lymphocytes, while in chronic lymphocytic leukemia patients it is present in approximately 20 %; but only present in a very small minority of HCV-related MC cases. Sequence analysis of the clonal variable Ig (Ig V) gene from MC and HCV-related B-cell NHL patients indicated a restricted expression of VH and VL genes (*VH1-69* and *VK3-A27*), suggesting involvement of a common antigenic epitope which, following its selection and expansion in a B-cell clone, can give rise to an overt lymphoma [96]. Previous studies have demonstrated that HCV-driven clonal expansion of B cells in MC, having a memory phenotype, leads to production of VH1-69 natural antibodies (Abs) and escape to control mechanisms [97]. In a fraction of investigated cases, these cells may replace the entire pool of circulating B cells, without varying their number. Thus, this may both subvert homeostasis of B-cells and provide genetic alterations causing additional growth and giving rise to an overt lymphoma.

The viral antigen(s) involved in B-cell clonal expansion have not yet been identified. In this regard, in monoclonal B-cell expansion of MC and in immunocytoma, the BCR in the monoclonal over-expanded B-cell subset, analogously to the IgM+RF cryoprecipitate, cross-reacts with the NS3 antigen of HCV [14, 94]. Another implication of HCV in lymphomagenesis has been provided by the binding of viral E2 envelope antigen to BCR cloned from an HCV-associated B-cell NHL. Therefore, in some HCV-associated B-cell NHLs and MC, it appears that B-cells respond directly to stimulation of viral antigens, since the Igs from one of the two tested HCV-associated lymphomas bound the E2 protein in a manner identical to human anti-E2 antibody [98].

Overall B-cell clonal expansion seems to be promoted by different HCV-related effects, including mutation/overexpression of B-cell proto-oncogenes, anti-apoptotic *BCL-2* by chromosomal translocation and interactions of HCV proteins with intracellular regulatory molecules, reviewed in [14], or with B-cell membrane receptors [70, 72], or binding of HCV-containing immune complexes to RF B- cells, as shown in mice [99].

There may also be an indirect role for HCV in LPD pathogenesis by means of host-immune response [100]. Indeed, in patients with chronic hepatitis C, B-cell functional activation was present in intrahepatic lymphoid follicles with GC formation, similar to those found in lymph nodes. HCV-associated immunocytoma can account for clonal proliferation of highly selected clones of B-cells that secrete cryoprecipitable RF, which is frequently encoded by the 51p1 VH gene combined with

the kv325 VL gene, thus confirming that an antigen-driven process supports a crucial role for clonal antigenic stimulation and evolution of B-cells in this lymphoma [101].

Treatment with E2-proteins or with viral particles produced from HCVcc, of cell line Raji, and of PBMCs, triggered IkBα phosphorylation; this led to overexpression of nuclear factor kappa B (NF-kB) and B-cell lymphoma-2 gene family proteins (BCL-2 and BCL-xL), and enhanced protection of both cell types from FAS-mediated cell death [102]. In addition, E2-CD81 signaling was accompanied by an increase in CD81 signaling and costimulatory CD80 and CD86, and a decreased in the complement receptor CD21.

Therefore, the E2-CD81 interaction may play a relevant role in HCV-induced LPDs and insufficient neutralizing immune response. Chronic antigenic stimulation plays a relevant role in initiating polyclonal expansion leading to genetic aberrations; however, the lack of ability of lymphoma BCR to bind HCV antigens in most of the investigated B-cell NHLs of patients with chronic viral infection supports the lack of generalization of this mechanism [103].

b) Chromosomal aberration in HCV-associated lymphomagenesis

Chromosomal translocations of immunoglobulin genes, which dysregulate oncogene expression placed under the control of Ig enhancers, are distinctive features of Ig gene remodeling in B-cell lymphomas [81]. Involvement of Ig loci in chromosomal translocation causes remodeling of Ig genes, during B-cell development, which generates antibody diversity by assembly of the V gene of the Ig heavy chain, through V(D)J recombination, somatic hyper-mutation (SHM) and isotype switching. Emblematic examples are the induced transcriptional dysregulation of B-cell lymphoma-1 gene *(BCL-1)* in mantle zonal lymphoma, *BCL-2* in follicular lymphoma (FL) and *MYC* in Burkitt's lymphoma, which are implicated in cell cycle, apoptosis inhibition and cellular growth regulation, respectively.

Chromosomal translocation 14:18 (t(14;18)) was also extensively observed in circulating B-lymphocytes from cases of chronic hepatitis C and MC [104-106], but not in liver biopsies from portal inflammatory infiltrates of HCV-infected patients [107]. This translocation is involved in malignant lymphoma, and is a transposition of the anti-apoptotic *BCL-2* gene, located in chromosome 18, joined to the 5' end of the IgH gene (IgH/*BCL-2*) on chromosome 14, causing overexpression of BCL-2 [108]. The most convincing evidence that HCV is directly involved in genetic changes observed in chronic viral infection and in benign HCV-associated LPDs, such as MC, is demonstrated by the disappearance of the t(14;18) translocation and Ig heavy chain (HC) gene rearrangement in most patients with chronic hepatitis after antiviral treatment [109]. This is reinforced by the reduced risk of developing lymphoma observed after obtaining a SVR following HCV treatment with IFN and RBV. Moreover, antiviral treatment is also able to abolish clonal B-cell expansion [18, 21, 85].

The t(14;18) translocation was firstly found associated with an increased frequency of the *BCL-2* gene rearrangement in cases of HCV-related MC [105, 109], with or without B-cell NHLs [110]; however this translocation was mainly found in follicular lymphomas, although in a low percentage (Table 1) [111]. The high-affinity binding of BCRs to HCV or to the envelope E2 protein [70, 71], can lead to proto-oncogene *BCL-2* activation by an increased frequency of t(14;18) [104, 105], compared to the frequency observed during B-cell development in uninfected immature B-cells of BM germinal centers to generate antibody diversity, reviewed in [81].

In HCV infected subjects the V(D)J rearrangement rate is amplified [104-106, 110], activating BCL-2 protein which is anti-apoptotic in B-cells due to a discrepancy of the BCL-2/BAX ratio, and enables survival of the anomalous B-cells. This is characteristic of the initial polyclonal expansion observed in MC, which represents an intermediate step before the development of frank B-cell NHL [104, 110].

B-cell expansion may stimulate production of autoantibodies, including the anti-IgG rheumatoid factor participating in IgG-M immune-complexes and MC [20]. Indeed, chronic antigenic stimulation plays an important role in the development of initial polyclonal B-cell expansion preliminary to genetic alterations, but a second hit, represented by altered expression of putative oncogene(s), can lead to overt lymphoma.

Remarkably, in these translocations t(14;18) the *BCL-2* gene is frequently found bound to the JH6 gene [112]. In particular, it has been observed that the major breakpoint region (MBR) of *BCL-2*, a 150-nt region of *BCL-2* exon 3 bound to a JH6 gene, was more often found in HCV-associated translocation t(14;18) compared to normal subjects. This suggests that, in the B-cell compartment of HCV patients, secondary D-to-JH rearrangements increase in frequency, and provide the molecular background for establishing most of the (14;18) translocations [112].

Since t(14;18) is eliminated after successful antiviral therapy [109], the reported anti-apoptotic effect of BCL-2 is implicated in progression to lymphomagenesis of HCV-infected B-cells, reviewed in [13, 14]. In chronic HCV-infection, aneuploidy increases with respect to the control [113]. Moreover, PBMCs from HCV patients and liver- derived hepatoma cell lines infected *in vitro* presented genetic instability associated with the low expression of retinoblastoma (Rb) protein, which is involved in cell cycle arrest when DNA abnormalities are detected. Mice that express the HCV Core protein also had low Rb expression, ruling out the notion that chromosomal aberration was only observed in tumor cell lines [114]. Cytogenetic analysis in primary splenocytes of these transgenic mice expressing Core protein showed at least twice the frequency of polyploidy compared to control mice. Lower Rb protein expression could avoid mitosis checkpoints contributing to cell polyploidy, thus promoting neoplastic transformation.

c) Direct HCV-infection of B-lymphocytes produces permanent genetic damage

Direct HCV-infection of B lymphocytes in chronic carriers may generate cellular transformation through hyper-mutations [14, 83-85], inducing permanent genetic damage and chromosomal alterations in B-cells.

Acute and chronic HCV replication has been shown to induce an enhanced mutation rate of immunoglobulin genes [115, 116] and proto-oncogenes [117] in B-lymphocytes, together with increased aneuploidy and polyploidy (Table 1) [113, 114]. The E2-CD81 interaction induces double strand DNA breaks and hyper-mutations of the heavy chain of Ig genes; in addition it lowers the affinity and specificity of HCV-specific antibodies, therefore enabling HCV to escape from immune surveillance [116]. A significant increase in mutation frequency found in IgHC, could also arise in *BCL-6, TP53* and β-catenin (*CTNNB1*) genes in *in vitro* HCV-infected B-cell lines, Raji, Ramos and the B-lymphoma derived JT [53], as well as in *in vivo* PBMCs, in lymphoma and HCC tissues derived from HCV-infected subjects [117].

However, contrasting evidence has been recently provided in this regard, demonstrating the absence of mutations in *CTNNB1* and *TP53* genes in naïve and memory B-cells from PBMCs of HCV chronic carriers, and in livers from HCV-infected patients [118]. Furthermore, both *CTNNB1*

and *TP53* mutated genes have been implicated in the development of HCV-associated HCC, bearing mutations in the target genes above reported [119, 120].

There was no significant difference in the mutation frequency for *BCL-6,* to which a SHM rate physiologically occurring in GC B-cells in the selected B-cell types infected *in vivo* by HCV is attributed, compared to B-cell subsets of healthy subjects [118]. These data reinforce a previous report, indicating, the absence of a significantly increased mutational rate in unselected PBMCs from chronically HCV-infected patients, with or without LPDs or lymphomas where, paradoxically a reduced mutational frequency was detected compared to healthy controls [121]. Further investigations are needed to evaluate to what extent the observed discrepancies depend on commitment, transformation state and modality of HCV infection of target lymphoid cells.

The reported permanent genetic B-cell damage, called "mutator B-cell phenotype", by the "hit-and-run" theory of cellular transformation is probably due to permanent genetic damages, caused by DNA double-strand breaks, inducing error-prone DNA polymerase, caused by activation-induced cytidine deaminase (AID), which also stimulates tumor necrosis factor α (TNF-α) production [115-117]. AID is overexpressed in B-cells of HCV infected individuals, suggesting that AID could play a crucial role in HCV-lymphomagenesis. In this regard, the CD19+ cell subset of-B cells from PBMCs significantly overexpresses numerous genes correlated to lymphomagenesis, such as AID [122].

The high rate of chronicization of HCV-infection can be explained by molecular mimicry; this is an important immune-evasion strategy that provides host survival and persistence for several viruses. Structural and sequencing analysis has confirmed that the E2 protein of HCV displays neutralization epitopes in HVR1, and indicates that HCV mimics immunoglobulins; alterations in the mimicry domain are associated with the loss of ability to escape immune regulation [123]. Immunoglobulins, which are the effectors of human immunity, are tolerated "self" antigens; since the N-terminal domain of E2 has epitopes within HVR1 that are antigenically and structurally similar to the human Ig variable domain, E2 is a tolerated antigen directly involved in escape and persistence of HCV.

d) Direct oncogenic role of HCV in B-lymphocytes

HCV can cause HCC and is probably associated with papillary thyroid cancer [18]; therefore it can be considered an oncogenic virus [14, 73]. However, the mechanism of HCV-oncogenesis remains unclear; HCV neither replicates by reverse transcription nor integrates in the host genome or codes for known oncogenes [10, 92]. It is extremely unlikely that HCV could activate insertion mutagenesis or acquire cellular oncogenes by genetic recombination [67].

Mutations, deregulations and post-translational modifications in tumor suppressor genes, *RB1, TP53* and β-catenin/Wnt pathways [119, 120, 124] and/or other genes [10] were detected in HCV-associated HCC. However, the long latency of HCV infection has made it difficult to establish a causal relationship between HCV infection and proto-oncogene activation by mutations. HCV replication has been unequivocally demonstrated in hepatocytes from chronically infected subjects [40], thus supporting the hypothesis for direct involvement of HCV in HCC development. However, since not all HCV-associated HCCs have detectable HCV RNA [125] it has been postulated that a "hit-and-run" mechanism must be involved in HCV-carcinogenesis [117].

Transgenic mice expressing the full HCV genome in B cells were shown to spontaneously develop B-cell NHL *in viv*o [88]; however, evidence that HCV transforms human B lymphocytes *in*

vitro is still lacking [17]. It cannot be excluded that HCV exerts a direct oncogenic potential through some of its proteins that are known to be oncogenic, such as Core and NS3/4A, and other nonstructural proteins, e.g. NS4B and NS5A, see [10] for review.

Apart from being involved in basic functions of HCV life-cycle, all these viral proteins are also able to interact with signal transduction pathways involved in regulating cellular growth and proliferation in hepatic and extrahepatic districts; they display key activities for metabolic networks in apoptosis, transformation and IFN-signaling [126]. The presence of HCV has been reported not only in B-cell lymphoma, but in a number of healthy B-lymphocytes, such as monocytes $CD14^+/CD19^+$ [47] and in the CD27+ subclass, which is more resistant to apoptosis, and has therefore been indicated as a possible subset for an HCV reservoir in chronic hepatitis C [127]. Since persuasive evidence for HCV-permissiveness of T-lymphocyte has only been provided for the CD5+ subclass [87], it appears that the mechanisms commonly involved in HCV-lymphotropism, whose alterations could be relevant in lymphomagenesis, should differ considerably from HCV-hepatotropism, which is not mediated by CD5 expression.

The TP53 family isoform DNp63 was also found overexpressed in lymphoma [127] and is known to be overexpressed in other human cancers. In B-lymphoma cell lines expressing the HCV Core protein, and in primary B-lymphocytes from LPD patients, DNp63 was upregulated, while DNp73 was downregulated, providing an essential requirement for cell growth. In addition, phosphoinositide 3-kinase (PI3K) was required for Core dependent DNp63 transcription upregulation, strongly suggesting the involvement of deregulated PI3K in HCV-related LPDs [128].

Among the HCV proteins, HCV Core is the most pleiotropic protein, playing preeminent role in different signaling pathways concerning cell viability and proliferation [10]. HCV Core protein (the component of viral nucleo-capsid) is a multifunctional protein: it is involved in lymph node development, enhances TNF-α induced apoptosis, and promotes cell proliferation during HCV infection [129] owing to its ability to bind the cytoplasmic domain of the TNF-receptor [130]. Recently, very high levels of soluble TNF-receptors I and II were found associated with HCV-dependent MC and B-cell NHL, strongly suggesting that they were actively involved in LPDs [131].

The HCV Core protein can also induce IL-6 in $CD14^+$ cells via Toll-like receptor 2 (TLR2) leading to increased B cell proliferation [132]. In addition to hepatocarcinogenesis [133], Core protein is closely linked to lymphomagenesis in transgenic mice [134]. Indeed, RNA expression of HCV Core protein in enlarged lymph nodes, the disruption of IFN-signaling, associated with lymphoproliferative stimulus by type II CD95, caspases and interleukins, were strongly involved in development of LPDs and frank NH lymphoma [135]. Molecular profiling of human B lymphocytes expressing HCV Core provided evidence for significant changes in the expression of gene categories regarding cell death/apoptosis, hematological malignancy, antigen presenting and processing [136].

HCV infection is associated with B-cell NHLs, specifically MZL and DLBCL, while chronic antigenic stimulation is the main determinant in MZL, whose direct oncogenic role is supported by *in vitro* studies (Table 1). *In vitro* expression of HCV Core protein and NS3 proteins have been shown to induce nitric oxide synthase (NOS) and reactive oxygen species (ROS) generation; these cause DNA repair damage and mitochondrial injury that may precede cellular transformation [137].

A recent study conducted *in vivo*, by *in situ* NS3 immunostaining in biopsies from DLBCL and MZL patients, showed a strong association between NS3 protein detection and high-grade lymphomas [138]. NS3 has been previously reported to transform mammalian cells *in vitro*, see [10, 126] for review.NS5A protein disturbs mitogen signaling pathways, thus allowing protection from

TNF-α and TP53; NS5A induces apoptosis and promotes tumor growth and is a potential viral BCL-2 homologue that interacts with BAX. NS5A also inhibits sodium phenyl butyrate-induced apoptosis in Hep3B HCC cells [139]. HCV Core protein and NS5A behave as transcriptional trans-activators for a number of cellular activities such as NF-kB, activating protein-1 (AP-1), serum responsive element (SRE) and signal transducer and activator of transcription 3 (STAT3).

NS4B is known to modulate NS5A hyper-phosphorylation and trans-activate IL-8, reviewed in [126]. Moreover, it induces an unfolded protein response and endoplasmic-reticulum (ER) overload response-dependent NF-kB activation, activates the extracellular regulated protein kinase (ERK) and c-JUN N-terminal kinase (JNK) signaling cascades, leading to stimulation of STAT3 and matrix metalloproteinase-2 (MMP-2) activity and BCL-2 expression [140].

Significant stimulation of signal transduction and STAT3, MMP-2 and BCL-2 activation occurs in PBMCs from persistently HCV-infected patients and in cultured cells [140]. The mechanism by which HCV regulates STAT3, MMP-2 and BCL-2 has been recently studied. HCV allows regulation of MMP-2 and BCL-2 by activating the STAT3 signaling cascade; conversely STAT1/2 is upregulated by ERK, JNK and protein kinase C (PKC) pathways. In particular, JNK mediates extracellular signal transduction to the nucleus, activating cell proliferation/apoptosis and differentiation, as well as stress-mediated signaling by transcription factors [140].

e) Signaling pathways potentially involved in HCV-lymphomagenesis

Evolution of viruses means that they can modulate host-cell signaling pathways, favoring their replication. These modulations are a consequence of the binding of the virus to its host-cell receptor, cross-interactions between viral and cellular proteins and stress caused by infection. Several viruses are known to activate RAS/ RAF/ MEK metabolic pathways during early infection. Investigating the relationship between activation of these pathways and HCV replication has enabled the conclusion that HCV infection *per se* activates RAS, RAF and MEK pathways, which in turn stimulates HCV replication (Table 1) [141], thus providing an example of an evasion mechanism of the host antiviral system from HCV. This effect is mediated by attenuating the IFN-JAK-STAT pathway and is accompanied by downregulation of IFN-stimulated genes (ISGs), while HCV-activation of major vault protein (MVP) suppresses virus replication through IFN-1 expression [142].

HCV infection is associated with lethal diseases, such as HCC and B-cell NHL, causing a serious global health problem. However, the pathogenic mechanisms of these neoplasms remain largely unknown. HCV activates multiple cell signaling pathways; the signal transduction network dependent on the regulation of HCV infection, and the molecular machinery triggering this regulation have been recently investigated [10, 126]

IL-6 is involved in the development of MCs and B-cell NHLs in HCV-infected patients by TLR2 stimulation [85]. Expression of B-lymphocytes stimulator/B-activating factor (BLyS/BAFF), a cytokine belonging to the TNF-family, in splenocytes of transgenic mice, is associated with the clinical progression of HCV infection and may activate NF-kB, JNF and ERK pathways, stimulating B-cell proliferation. Investigations of the polymorphism of the *BAFF* gene promoter, such as the allelic variant -817, which correlates with an increased BAFF transcriptional activity [14, 143], have revealed a more frequent *BAFF* gene over-expression in sera from HCV-positive subjects with MC than in controls without MC [144, 145]. In addition, increased levels of osteopontin and IL-1β were observed in sera from patients with HCV-positive B-cell NHL. In HCV-positive subjects, the highest levels of osteopontin and IL-1β were closely associated with MC, regardless of B-cell lymphoma

development [146-148]. Expression profiles of lymphoma B-cells from HCV-transgenic mice displayed an altered expression of various genes [14, 84, 89, 126]. In particular microRNAs may act as effective biomarkers of DLBCL [149].

The NOTCH signaling pathway was deregulated in HCV-positive DLBCLs. Comparative analysis indicated that NOTCH2-mutations were found in 20% and NOTCH1-mutations in 4% of the HCV-cases compared to only 1/64 of HCV-negative ones [150]. PTEN mutations were only detected in 2% of cases. These mutations were associated with poor overall survival and histopathological alterations typical of DLBCL cell transformation. Since NOTCH1 is involved in the pathogenesis of splenic marginal zone lymphoma (SMZL), these data fit well with the reported observation that a high proportion of DLBCL in HCV-positive subjects can arise from a preceding MZL [18].

f) B-cell lymphoproliferation mediated by cytokines and chemokines

Numerous experimental evidence, conducted *in vivo* and *in vitro*, unequivocally supported that HCV replication takes place in B cells, reviewed in [17]. In these cells, direct involvement of HCV RNA (-) and the viral NS3 protein expression in HCV mRNA replication, translation and polyprotein production was demonstrated.

However, the principal HCV-target population of the immune system is still unknown. Major mechanisms involved in HCV-associated lymphomagenesis were found to be: induction of cytokines IL-2, IL-10 and the soluble IL-2R (sIL-2R), caspases 3/7 and BCL-2 in relation with the intracellular expression of oncogenic viral NS3 and Core proteins, as well the role played in activating IL-12 by HCV-E2 [14, 18] (Table 1). Although recent studies have strongly supported the oncogenic role of the HCV genome or some of its viral proteins in B lymphocytes [84, 85], the potential HCV penetration and replication in B lymphocytes still remain to be clarified. Penetration seems to be restricted to the CD5+ subset of the B-cell population, which also overexpresses CD81 [86]. It is also possible that for full permissiveness of B cells, other events, such as the EBV coinfection, may be required [83].

The immune response is modulated in particular by cytokines and chemokines, whose balance between activation and repression may induce several autoimmune diseases. Recent evidence has highlighted the role of cytokines and chemokines in the pathogenesis of HCV-related LPDs [14]. In both normal and leukemic B-lymphocytes IL-2R (Tac/CD25) expression is upregulated by IL-10 [151]. Consequently, both IL-2R and IL-10 may be involved in B-cell transformation of B-cell NHL, while BLyS/BAFF cooperate to induce the B-cell survival observed in LPDs.

Soluble interleukin receptor-α (sIL-2Rα), is involved in several types of cancers [152], including T-cell lymphomas [18]. In fact, sIL-2Rα overexpression in splenocytes correlated with a high incidence (25%) of DLBCL development in HCV-transgenic mice (RzCD19Cre), which express the full-length HCV-genome, under CD19-gene transcriptional control, in B-cells [89].

The role for cytokines and chemokines has been supported by recent studies, mainly in MC, where Th1 cytokines, such as IFN-γ and TNF-α and other chemokines (MIP-1α, MIP-1β, CXCR-3, CXCL10 and CXCL13) are highly expressed, as observed in vasculitis lesions, signifying a potential implication in pathogenesis [153]. CXCL13, which is required for B-cell trafficking, is another chemokine upregulated in MC [154], being overexpressed in liver biopsies from active cutaneous vasculitis. Since HCV-related MC patients with autoimmune thyroiditis present high serum levels of

CXCL10 and CXCL11, their role played in the MC pathogenesis should be investigated in depth [155].

THE IMPACT OF THE MICROENVIRONMENT AND miRNAs ON HCV-LYMPHOMAGENESIS

The role played in lymphocyte transformation via disruption of cell activity, uncontrolled cell division and immune function by environmental factors, such as inflammatory cells and soluble mediators leading to the development of indolent and aggressive B-cell lymphomas, has been the subject of extensive investigations in the field of lymphomagenesis and related viruses [156-158].

On one hand, it strengthens the causal correlation of HCV in many types of B-cell NHLs [18], on the other it raises the unsolved question of which other factors, e.g. in the environment, or those strictly involved in signal transduction pathways, impair basic cell functions in steps progressing along lymphoma development [158].

The interaction between lymphoma and the microenvironment helps explaining the geographical variability of HCV-induced B-cell NHLs [159]. It has been underlined that these imitate the regulated coexistence existing among malignant cells and the microenvironment, in analogy to the normal interaction between the latter and B-cells. The malignant development of the tumor cell growth requires external signals from the microenvironment namely the involvement of virus antigens, cell-cell interactions, cellular cytokines (IL-6, IL-7, IL-10, TGF-β), chemokines (CXCL1, 10, 11, 13) and caspases (Table 1) [18, 132, 147, 151, 155].

In this process, collaborating the lack of surveillance induced by viruses carrying immune deficiency, and immune stimulation dependent on lymphocyte activity by unknown or re-emergent factors, viruses are enclosed [158], possibly by involving a “hit-and-run” mechanism, as postulated for the HCV, susceptible to inducing hyper-mutations in Igs and proto-oncogenes [117].

Mutually non-exclusive pathogenic hypotheses were proposed to explain HCV-lymphomagenesis, after an initial "switch" of B-cell clonal proliferation by HCV antigenic stimulation, followed by chromosomal aberrations acting on the 14;18 translocation and *BCL-2* gene activation. Further costimulatory or successive "hits" should cooperate to drive infected B cells toward full malignancy. In addition to the described hyper-mutations in Igs and oncogenes/anti-oncogenes, which are under direct viral infection stimulus-by E2-CD81 binding at the cell surface, displaying transforming potential mediated by NOS, Core and mutagenic NS3/4 viral proteins, miRNA (or miR) dysregulation also plays a role (Table 1) [85, 149].

The role of miRNAs in gene silencing has recently been characterized in the eukaryotic gene regulation [159]. These miRs are highly conserved; they act as post-transcriptional regulatory elements that control gene expression by complemental base pairing at the 3’-UTR binding of target mRNA transcripts, enabling degradation or suppression of translation. Since a single miRNA targets multiple mRNAs, it is able to regulate the expression of many genes; miRNAs have a high tissue specific expression pattern and were found actively involved in organogenesis and carcinogenesis [160, 161], as well as viral pathogenesis and immunity [162].

Furthermore, several oncogenic DNA viruses code for miRs, thereby modulating the regulation of several cellular genes functional for their replication; nevertheless, this does not happen for HCV and other RNA viruses linked to infection and cancer. However, a new class of virus-

derived small RNAs (vsRNAs), showing very high homology to infectious viral genomes, has been described [158].

miRs modulate host-cell mRNA expression leading to important phenotypic changes of cell inflammation, differentiation and proliferation, favoring pathological processes leading to neoplastic transformation. One example is the hepatic specific miR-122, which regulates pathways that maintain liver-specific functions [161], and promotes HCV RNA replication [39]. Upregulation of miR-155 has been associated with NHLs and other solid and hematologic malignancies; the miR-17-92 cluster is specifically involved in B-cell lymphoma, while miRNA-143 and miR-145 expression are reduced [158]. It has been suggested that miRs are involved in activating both canonical and alternative NF-kB pathways [163], and down-regulation of miR-26b may contribute to lymphomagenesis. Reduced miR-26b expression has been demonstrated in HCV-positive patients with SMZL [85], in whom total subversion of the microRNA pattern was observed [164]. A significant decrease in miR-26b expression was also detected in PBMCs from MC and NHL patients, accompanied by an increase in miR-21, miR-16 and miR-155 expression, in NHL only [165]. The reduced expression of miR-26b accompanied the decrease in levels of miR-139, 345, 125a and 126 [164]. Overexpression of miRs 21, 155 and 146a was shown in splenic tumor tissues from SMZL patients, compared to normal controls. A decrease in miR-138-5p expression, together with a greater expression of miR-147a/miR-147b and miR-511-rp, is related to poor prognosis in HCV-positive DLBCL [149].

ANIMAL MODELS FOR INVESTIGATING HCV- LYMPHOMAGENESIS

In the last decade, transgenic mice have been successfully used to investigate HCV-lymphomagenesis [166]. Humans and primates are the only species susceptible to HCV infection and develop persistent viral infection. More recently, a genetically-humanized rodent model and xenotransplanted and hepatic chimeric mice were very promising for investigating the signaling pathways and molecular steps involved in this form of virus-associated lymphomagenesis [14, 18, 72, 78, 84, 114, 126].These alternative animal models are useful in investigating the characteristics of the HCV replication cycle and steps of viral infection.

HCV-lymphomas are heterogeneous [18, 73, 75]; different oncogenic pathways may be predominantly involved, according to the subtype [83-85, 167], and transgenic mice are therefore important to discover the mechanisms implicated in pathogenesis.

Viral replication in lymphocytes was demonstrated in patients with HCV-chronic infection with or without MC [11, 15-17, 43], and the involvement of BM was shown [44-46]. Cumulative data then correlated the presence of HCV in lymphoid districts with autoimmune LPDs in humans [12-14, 41, 42, 72]. This was highlighted in a study investigating HCV-persistence in *in vitro*-infected human lymphoid cells injected into SCID mice, which revealed the presence of HCV RNA in serial B-cells sub-passaged in these animals [88]. HCV RNA - it-self - may chronically stimulate the immune system to induce B-cell NHL in mice [89]; as well as other viruses, see [14] for review.

Transgenic mice expressing in their splenocytes BLyS/BAFF, a cytokine belonging to the TNF-family, had clinical progression of HCV infection. Moreover, several pathways stimulating B-cell proliferation were activated (NF-kB, JNK and ERK), reviewed by [85]. To exclude that chromosomal aberrations were only observed in cancer cell lines, mice expressing HCV Core protein were studied by cytogenetic analysis; their primary splenocytes displayed nearly twice the frequency

of polyploidy than the control mice [100]. Lower Rb protein expression could avoid mitosis checkpoints contributing to cell polyploidy, thus promoting neoplastic transformation. The HCV Core protein in transgenic mice was shown to induce HCC [133], and malignant lymphoma [134, 168]. The expression of HCV Core RNA in enlarged lymph nodes and the observed disruption of INF-signaling were strongly related to the development of LPDs and frank NH lymphoma.

Both IL-10 and IL-2R could be involved in B-cell transformation. Functional IL-2 receptor Tac/CD2 expression is upregulated by IL-10 in normal and leukemic B-lymphocytes [151]. Indeed, transgenic mice RzCD19Cre expressed high levels of sIL-2Rα in B-cell splenocytes and in 25% developed DLBCL. These mice contain the full-length HCV-genome under the B lineage-restricted CD19-gene transcriptional control [89, 168], strongly supporting the evidence that persistent HCV expression could be directly involved in B-cell lymphomagenesis. To investigate the signaling pathways of HCV-associated B-cell NHLs, comprehensive molecular analysis was performed in HCV transgenic mice RzCD19Cre using a genome-wide microarray [163]. Expression profiles of lymphoma B-cells from HCV-transgenic mice displayed an altered expression of various genes, such as *FOS*, *C3*, *LT R, A20,* miR-26b and *NF-kB*; this suggested that activation of both canonical and alternative NF-kB pathways and down-regulation of miR26b actively contribute to lymphomagenesis, since reduced expression of miR-26b has been found in HCV-positive SMLZ patients [164].

There was a correlation of tumor volume with the concentration of tumor-derived soluble IL-2R in body fluids of host SCID mice xenografted by human T-cell lymphoma [169]. Elevated sIL-2R serum levels in lymphoid malignancies correlate with the clinical stage of disease. A second murine model is the Irf1--/CN2, in which HCV-structural proteins (E1, E2, NS2 and Core) disrupted IFN regulatory factor-1. These mice have a high prevalence of lymphomas and LPDs, as well as increased levels of IL-2, IL-10 and BCL-2 together with BCL-2 expression. Therefore, in this mouse model, overexpression of apoptosis-related proteins and/or aberrant cytokine production was the primary event involved in inducing lymphoproliferation [168].

Functional proof that genes associated with leukemia/lymphoma were under HCV Core upregulation was reported in mice, in which the core gene was expressed by an adenoviral vector [136]; apoptosis was inhibited and there was a dramatic down-regulation of MHC class II in primary B-lymphocytes. Suppression of FAS-mediated cell death and apoptosis was observed in a transgenic mice model expressing Core, E1, E2, and NS-2 HCV-proteins under the control of the CD2 promoter [170]. In these mice, release of cytochrome C from mitochondria was abolished by expression of viral proteins that inhibited activation of caspases 9 and 3/7, but not caspase 8, thus indicating that these caspases are the targets of above viral proteins implicated in the suppression of FAS-mediated cell death and apoptosis.

HCV Core protein -mediated injury in transgenic mice liver is driven by reduced IFN γ and IL-2 production, similarly to that in chronic HCV-infected subjects, indicating interaction of FAS-mediated apoptosis in the liver infiltration of peripheral T-cells [171]. A similar mechanism of core expression in T-lymphocytes may play a crucial pathogenic role in immune dysregulation by liver T-lymphocyte infiltration and injury.

CONCLUSIONS

The mechanism(s) of HCV-lymphomagenesis remain, in part, elusive. Some pathogenic features of HCV-associated LPDs and B-cell NHLs have been clarified [18, 85]: 1) virus lymphotropism and 2) immune escape in the close association of HCV with MC and B-cell NHL [11-13, 17, 172, 173], and some of the major virus/host factors involved have been identified [157-159].

Direct and indirect mechanisms in HCV lymphomagenesis have been widely reviewed [14, 19, 20, 42, 72, 73, 78, 84, 127, 168, 174] namely anti-apoptotic mechanisms, and clonal expansion. The latter is mediated by viral-modulation of growth regulatory signals and oncogenic pathways, through the hyper-expression and mutation of oncogenes/immunoglobulins and virus/host-induced immune evasion [41, 71, 92-96, 98, 102, 110, 114-117, 135, 137, 138, 143, 149, 165]. These result in uncontrolled cellular growth, including full malignant transformation [83, 130, 131, 140, 141, 149, 150, 163, 165, 175]. The different oncogenic mechanisms implicated appear to be mutually non-exclusive [83]. Their order, and how they may interact and integrate reciprocally to produce a synergic oncogenic stimulus, sufficient to drive HCV-infected B-cells toward lymphomagenesis, has not yet been entirely clarified in most known B-cell NHL subtypes [18], (reviewed in [175]).

The "*primum movens*" is the HCV-antigenic chronic stimulation of B-cells [88, 89, 92, 103] due to HCV-E2 binding to the CD81 complex and/or HCV-specific antigens to BCR [70-72, 98]. This binding results in a reduced threshold for B-cell activation and proliferation [90, 91] leading to abnormal cell growth and polyclonal expansion, preferentially in the MZL subtype and occasionally in MZL-derived DLBCL [167]. The E2-CD81 interaction induces hyper-mutations in the Ig gene of HCV-infected B-cells [115]. The mutation rate of the VH gene in Ig of B cells producing antibodies may lower the affinity and specificity of HCV-Abs, enabling HCV to escape from host immune surveillance [116].

The progression from pre-neoplastic LPDs and indolent benign low-grade lymphomas toward overt malignant B-cell NHLs has been suggested to occur via chromosomal translocations t(14:18) [104-106, 110]. This event causes cell proliferation through *BCL-2* gene hyper-expression [85], especially in the FL subgroup, but also in carriers of other HCV-associated B-cell NHL subtypes and MC [167]. The evolution of HCV-unassociated FLs was probably driven by GC reentries of B-cells overexpressing BCL-2, as demonstrated in mice [176]. In this case, the non-cognate bystander effect, which occurs in GC in HCV-unassociated FL and consists of T-cell help, primarly based on CD40 and cytokines, has been postulated to arise outside GC, involving other different mediators [167].

The "hit-and-run" theory in HCV lymphomagenesis [115-117], involves genetic mutations in known anti-oncogenes induced in HCV-infected liver cells, and was hypothesized but not demonstrated for hepatocarcinogenesis [119, 120]. Thus, involvement of this pathogenic mechanism in HCV-associated B-cell NHLs requires additional *in vivo* confirmation [118, 121].

In cases of HCV-positive DLBCL, translocations of *MYC* and/or *BCL-6* were detected, together with altered protein expression (CD37, IgA-G-M, MYC, BCL-2) and mutations of TP53 in tumor tissues [177]. Even patients with occult HCV infection presented greater expression of lymphoma-associated genes such as *AID*, *BAL*, cyclin D1 and 2, serine-*STK15* and *Gal-3* indicating that these genes are upregulated after viral therapy, in particular if HCV is detectable in PBMCs [178].

All the described theories indicate a crucial role for dysregulation of the microRNA network, since miR-26b downregulation is involved in weakening tumor suppression [164, 167].

The therapeutic potential of antiviral IFN+RBV therapy in low-grade HCV-related B-cell lymphomas demonstrates the role of HCV in the disease [19, 21]. However, the same therapies were ineffective in most patients with more aggressive lymphomas, such as DLBCL, associated with HCV in chronically infected subjects [18, 78, 179]. Two groups of DLBCL have been identified; namely *de novo* DLBCL, which is homogenously composed of large B cells; the second group is characterized by small B-cell infiltration originating from MZL transformation into the more aggressive DLBCL [167]. Thus, also in DLBCL patients, the occurrence of an additional evolution from benign low-grade B-cell NHL toward malignancy was driven by successive mutations in oncogenes/anti-oncogenes and/or genetic/epigenetic changes due to environmental factors [157-159].

B-cell NHLs are of different pathogenic grades and present diverse molecular targets. The therapeutic response in individual cases can therefore differ. In any case, HCV predisposes to all the direct or indirect oncogenic potential to interact with host B cells to reach a pre-neoplastic lymphoproliferative state, prior to full malignant transformation. Permissiveness to infection and commitment of B cells to transformation require further considerations [167].

The genetic predisposition in the HCV-induced HCCs and B-lymphomas is supported by accumulating evidence. Single-nucleotide polymorphism (SNP) analysis in the HLA class II *HLA-DRB1/DQA1* and *NOTCH4* genes revealed a close association with benign and malignant LPDs related to HCV infection. A genetic variant of *TNFAIP3/A20* (the *rs2230926G* allele), was more frequently found associated with patients with RF activity independently from the histological lymphoma subtype. Therefore, a minor innate A20 dysfunction causing NF-kB activation may result in the escape of autoimmune B-cells during chronic stimulation [167].

The HLA/KIR profile was investigated in HCV-related disease progression; KIRDS2 and KIR3DL2 variants were found in both benign and malignant HCV-associated LPDs. HLA-Bw6/KIR3DL1 combination of KIR and HLA haplotypes displayed an increased risk of developing lymphoma compared to MC; the HLA-Bw4/KIR3DS1 was also related to an augmented risk of HCV-associated HCC [180]. HCV chronic carriers have different probabilities of progression to severe liver damage or into LPDs depending on the presence of haplotype rs12979860 IL28b TL2R-174, demonstrating that innate immunity plays a role in HCV disease progression [181].

The genetic and molecular signatures of the diverse forms of HCV-related B-cell NHLs were considered in order to understand their pathogenesis [83-85]. They combined specific signatures and expression of different sets of genes associated with BCR expression, coupled with BLyS signaling; the latter was associated with the control of B cell maturation and NF-kB transcription factors [182]. The high heterogeneity associated with DLBCL malignancy can also result from *de novo* transformation of other more benign B-cell NHLs [78].

Depending on different genetic profiles, DLBCL can be of two subtypes: one derived and developed through GC reaction and the other from distinct phases of B-cell differentiation [156].

Proteomic analysis of B-cell lymphoma [183] helped classifying the HCV related one, based on protein expression profiles [167].

Expression profiles of lymphoma B-cells in HCV-transgenic mice displayed an altered expression of various genes [182], linked to activation of both canonical and alternative NF-kB and other oncogenic pathways. Gene expression profiling of B cell malignancies unrelated to HCV also allowed characterization of two distinct molecular forms of DLBCL; the first one expressed genes

characteristic of the GC, the second expressed genes normally induced during activation of B-cells *in vitro* [184]. Both showed pathways of different differentiation stages, based on their gene expression pattern, partly reflecting their tumor differentiation and proliferation rates, which are predictive in the host of clinical responses and outcomes [183, 185, 186].

B-cell NHL associated with MC vasculitis in HCV carriers can be identified on the basis of the signature of sCD-27, sIL-2R and gamma-globulin C4 levels, with diagnostic and prognostic potentialities [187].

All these mutually non-contrasting mechanisms drive the progression toward malignancy through different signaling pathways and successive development steps [83-85], and cooperate in the pathogenesis of HCV-lymphomas together with environmental factors [157]. The wide biological heterogeneity of HCV chronic carriers makes it difficult to predict which patients will progress to malignancy [188]. A prognostic and predictive early evaluation of therapeutic responses and outcomes of these tumors is crucial to antitumor therapies [73].

The recent success of DAAs [7-9, 79] in curing HCV infection needs further in-depth and longer clinical evaluation for assessing their effect on eradicating the different subtypes of B-cell NHLs. Treatment for lymphomas, moreover, could be ameliorated by novel therapeutic options such as CAR-T cell therapies [189, 190]. The anti-lymphoma DNA vaccines, targeting anti-idiotypic immunoglobulins, are so far limited to mouse models [191-193] although human vaccine trials have been initiated [194].

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Conflicts of interest statement

The authors declare they have no conflict of interest.