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Reversion of anergy signatures in clonal CD21^{low} B cells of mixed cryoglobulinemia patients after clearance of HCV viremia with Direct-Acting Antivirals

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Chapter 1

INTRODUCTION

Hepatitis C virus (HCV) infection represents a major public health problem and is considered as one of the most lethal infectious diseases next to influenza, respiratory syncytial virus, rotavirus, hepatitis B (HBV) and human immunodeficiency virus (HIV) [1,2]. The World Health Organization (WHO) estimates that at least 130-150 million people, approximately 3% of the world's population, are chronically infected causing about 700,000 deaths every year [3]. Chronic HCV infection may lead to hepatic fibrosis and eventually cirrhosis, at which stage patients have a substantial risk of liver failure, hepatocellular carcinoma (HCC) and liver-related death; moreover, HCV infection is associated with several extrahepatic manifestations which increase the non-liver-related mortality rate and have a strong impact on health-related quality of life and on direct and indirect health care costs [4,5].

Over the last few years this viral disease even gained prime focus in medicine in general; this momentum of chronic HCV infection results from the success story of antiviral treatment development. Interferon-based antiviral therapy, aimed at immunomodulation to inhibit HCV replication, has been a treatment option for over two decades; in the recent five years, new interferon-free antiviral drugs, called direct-acting antivirals (DAAs), have been developed and revolutionized the treatment of HCV because they are much more effective, safer and better-tolerated than the older therapies [6].

1.1 An overview of HCV: origin, epidemiology and diversity

HCV is a *Hepacivirus* that belongs to the *Flaviviridae* family, first discovered in 1989 by Choo and coworkers from the serum of a patient with non-A, non-B hepatitis [7]. Although its origin remains unclear, this virus might be originated from zoonotic sources such as non-human primates (e.g. monkeys, apes) and mammals (e.g. dogs, horses) [8].

A single HCV particle display a diameter of approximately 68 nm and it consists of a core of genetic material, surrounded by an icosahedral protective shell of proteins further encased in a lipid envelope of cellular origin [9]. The viral genome is made up of a linear positive-sense single-stranded RNA, whose total length is about 9.6 kb, with one open reading frame (ORF) and 5′ and 3′ untranslated regions (UTRs) at both edges (Fig. 1) [10].

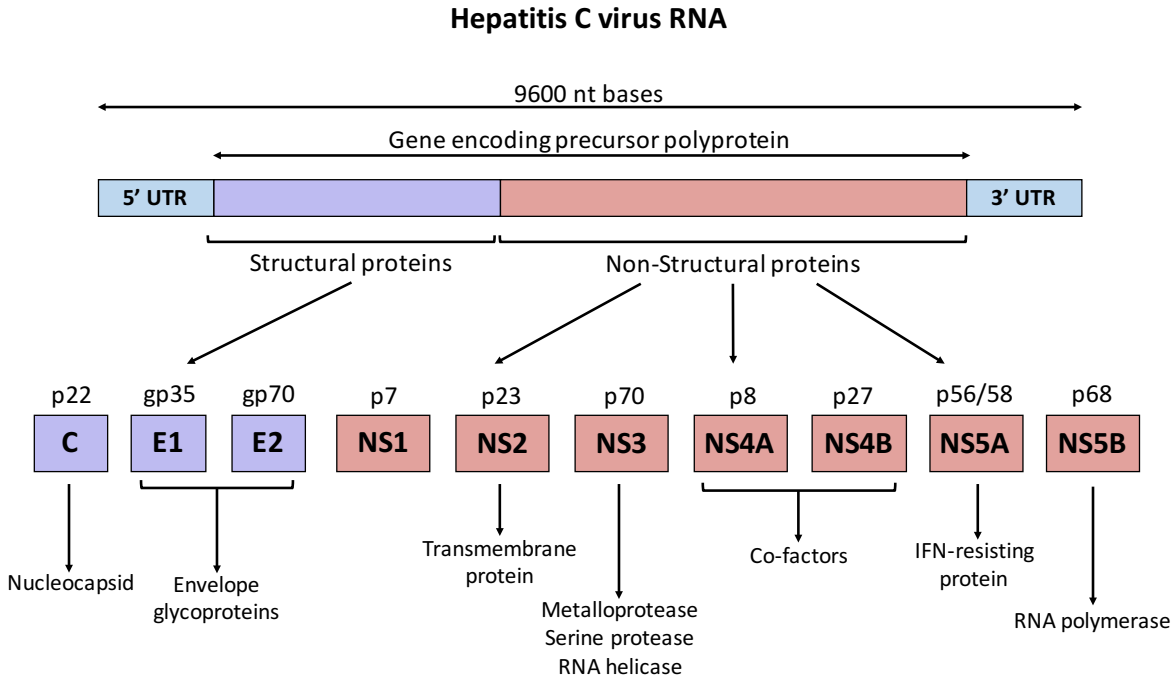


Figure 1. HCV genome. *Structural and non-structural proteins encoded by HCV RNA*

The open reading frame is translated to produce a single protein product (polyprotein) which is then cleaved, by viral and cellular proteases, into 10 structural and non-structural (NS) proteins: the first group, including core protein (C) and the envelope proteins E1 and E2, constitutes the skeletal structure of the virion, while P7, NS2, NS3, NS4A, NS4B, NS5A and NS5B, that are part of the second group, act as enzyme or regulatory factors that are essential for viral replication. Core protein modulates gene transcription, cell death, cell proliferation and interference metabolism leading to oxidative stress, liver steatosis and finally hepatocellular carcinoma (HCC); HCV envelope proteins E1 and E2 are generally glycosylated and have played a major role in cell entry; protein P7 is responsible for ion channel and virus assembly [11].

Among non-structural proteins, NS3 functions as a serine protease and forms a heterodimeric complex with NS4A that acts as a cofactor of the proteinase; the NS2 cysteine auto-protease and the NS3/4A serine protease are responsible for the cleavage of downstream, non-structural proteins, including NS5A. NS5A is a hydrophilic phosphoprotein which plays an important role in viral replication, modulation of cell signaling pathways and the interferon (IFN) response. The NS5B protein is the viral RNA dependent-RNA polymerase, which catalyzes the polymerization of ribonucleoside triphosphates (rNTP) during RNA replication. Given the critical role of NS3/NS4A, NS5A and NS5B in the viral life cycle, they are currently focused on as major targets in development of DAAs.

RNA replication takes place in distinct compartments of the cell cytoplasm and requires both viral (NS3-NS5) and host proteins. During replication, HCV genomic RNA is transcribed into a complementary RNA strand, which subsequently constitutes a template for the synthesis of new viral genomes [12]. Owing to the highly error prone of the RNA polymerase, and to the resulting low fidelity of the genomic RNA replication, a naturally high rate of genetic mutation generates genetic diversity of HCV. Based on the sequence variation of different HCV strains, it is possible to identify 7 genotypes (genotype 1-7) with several subtypes within

each of them, whose global prevalence is unevenly distributed: in particular, genotype 1 (46,2%) and 3 (30,1%) dominate the global infections; genotypes 2, 4 and 6 characterize about 22,8% of HCV infections; genotype 5 accounts for the remaining less than 1% [13] and finally genotype 7 has been identified so far in very few patients originating from Central Africa [14]. Interestingly, the nucleotide diversity of HCV genomes is approximately 32,4% between virus genotypes and 14,6% within each of them [15]. Genotype is clinically important in determining potential response to therapies; genotypes 1 and 4, for example, are less responsive to IFN-α-based treatment than are the others [16], and this leads to difficulties in clearing HCV infection of these two genotypes. On the other hand, the latest developed Direct-Acting Antivirals (DAAs) have the potential to eradicate all genotypes of HCV, hence extending the patient population who may benefit.

1.2 HCV-related lymphoproliferative disorders

Patients with chronic HCV infection are known to be at risk of developing liver complications, such as cirrhosis and hepatocellular carcinoma (HCC), but the morbidity and the mortality are underestimated because they don't take into account the extrahepatic manifestations of HCV infection. Indeed, although hepatocytes are the primary target, early after its discovery it was shown that this virus is not only hepatotropic but also lymphotropic [17]: Muller et al. first reported in 1993 that HCV RNA could be found in B cells [18] and they predicted that peripheral blood mononuclear cells (PBMCs), particularly B cells, could be sites for HCV replication and may serve as reservoirs of HCV infection; many subsequent studies have confirmed that HCV may also infect PBMCs [19-21] as indicated by the presence of HCV RNA, as well as structural and non-structural viral proteins, in PBMCs of chronically HCV-infected patients or in perihepatic lymph nodes [21-23]; other studies have reported the ability of serum-derived HCV particles to infect PBMCs [24,25] or particular human T-cell leukemia virus type 1- infected cell lines

[26]; finally, a study reported the establishment of a B-cell line deriving from a HCV-infected patient non-Hodgkin's B-cell lymphoma (B-NHL) [27].

As a consequence of the lymphatic infection, several lymphoproliferative disorders (LPDs) have been associated with this virus [28], including Type II Mixed Cryoglobulinemia (MC), B-NHL [29,30] and monoclonal gammopathies [31].

A number of evidence suggests that the way through which HCV causes LPDs is represented by the protracted antigenic stimulation [32,33]. Interestingly, this hypothesis is strengthened by the preferential usage by the monoclonal B cells expanded in HCV-related LPDs of a restricted set of cross-idiotypes (XId), commonly encoded by IGHV1-69, IGKV3-A27 and IGKV3-20 heavy chain variable genes [34-37].

It has been postulated that HCV proteins may facilitate B cell activation through different mechanisms: one of these is the binding between HCV envelope protein E2 and the tetraspanin CD81 expressed on B cell surface, that leads to the formation of a co-stimulatory complex with CD19 and CD21, induces a decrease in B cell activation threshold and subsequently triggers the JNK pathway leading to B cell proliferation [38]; another mechanism is the interaction of the HCV nonstructural protein NS3/4A with the DNA damage sensor CHK2, leading to a cascade of events that upregulate BCR signaling [39]. Conversely, HCV infection of memory B cells through the binding of E2 and 5'-UTR to the

B7.2 (CD86) co-receptor inhibits differentiation into plasmablasts and immunoglobulins (Igs) production [40].

Interesting data are available about the role played by chromosomal aberrations in HCV-related LPDs. The most investigated genetic aberration was the (14;18) translocation, that was found to be significantly associated with type II or monoclonal MC. The presence of this translocation in MC was correlated with the overexpression of the anti-apoptotic *bcl-2* gene in B cells, resulting in an imbalance of the Bcl-2/Bax ratio and abnormal B cell survival [41,42] (Fig. 2). It has been reported an increased rate of aneuploidy in chronically infected HCV subjects

versus healthy controls, with values similar to an NHL group. This observation suggests that HCV patients could be more prone to develop a lymphatic malignancy also because of bearing such alterations of the ploidy grade [43]. Finally, a growing body of evidence support the key role of cytokines and chemokines [44], as well as of microRNA (miRNA) [45], in the pathogenesis of chronic HCV infection and related extrahepatic disorders (Fig. 2).

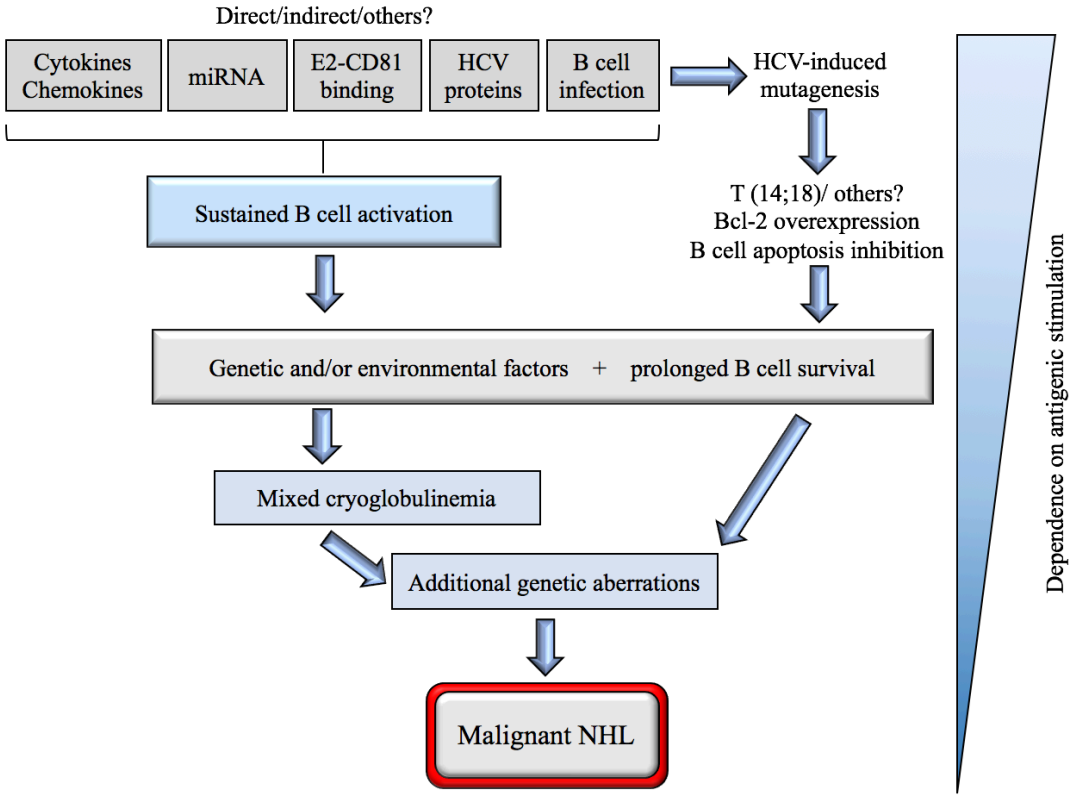


Figure 2. HCV-related pathogenesis is a multifactorial and multistep process.
Current data suggest that the starting points of this process are represented by the cooperation between a sustained and persistent stimulation, by direct or indirect action of viral particles or proteins, and anti-apoptotic mechanisms acting on B cell compartment. A predisposing genetic background would be responsible for the final evolution to a particular LPD (namely, mixed cryoglobulinemia). The progressive addition of genetic aberrations would lead to a frank neoplastic transformation, gradually making the process less dependent on the etiologic agent [41-45].

1.3 Cryoglobulinemia: physical properties of cryoglobulins, diagnosis and laboratory evaluation

The term "cryoglobulinemia" refers to a clinical condition characterized by the presence of immunoglobulins in serum that precipitate, *in vitro,* at temperature below 37°C and redissolve after rewarming; these cold-precipitable immunoglobulins were first identified by Wintrobe and Buell in 1933 and subsequently named "cryoglobulins" by Lerner and coworkers in 1947 [46]. Interestingly, cryoglobulins are not necessarily a sign of disease since healthy people may have low concentrations of them and polyclonal cryoglobulins may be transiently detected during infection [47].

In 1974, Brouet et al [48] proposed a classification of cryoglobulinemia that is still widely used due to its good correlation with clinical features and associated diseases:

- monoclonal cryoglobulinemia (type I)
- mixed cryoglobulinemia (type II and type III)

In Type I cryoglobulinemia, cryoglobulins, which account for 10-15% of the total, are composed of a single monoclonal cryoprecipitable immunoglobulin (mainly IgG or IgM and rarely IgA or free immunoglobulin light chains [49]) produced by the monoclonal expansion

of a B cell clone that may be indolent in about 40% of patients (monoclonal gammopathy of unknown significance [MGUS]) or overtly malignant in the remaining 60% (Waldenström macroglobulinemia [WM], multiple myeloma [MM] or chronic lymphocytic leukemia [CLL]) [50]. In type II cryoglobulinemia, cryoglobulins, which account for almost 50-60% of cases, are characterized by a polyclonal component, mainly IgG, mounting k or λ chains, and a monoclonal IgM (rarely represented by IgA or IgG) endowed with rheumatoid factor (RF) activity. Most IgM react with both intact IgG and the $F(ab)_2$ fragment and also with the Fc

fragment of autologous IgGs, and these two types of molecular interaction confer greater stability to cryoprecipitating IgM-IgG immune-complexes. This type of cryoglobulinemia is usually associated with HCV infection in up to 90% of patients [51]; different causes include other infections (mainly HIV and HBV), connective tissue diseases (CTDs) and lymphoproliferative disorders. Approximately 10% of patients have no identifiable cause and in this case it is referred to as "essential mixed cryoglobulinemia" [52]. Type III cryoglobulinemia (25-30% of cases) is characterized by polyclonal IgM with RF activity and polyclonal IgG and it's seen in CTDs or secondary infection (mainly HCV). However, there are unusual cryoglobulins showing a micro-heterogeneous composition, whose immunochemical structure cannot be fitted into any of the categories described above. Using sensitive methods, such as immunoblotting or two dimensional polyacrylamide gel electrophoresis, some authors described MC formed by oligoclonal IgMs and traces of polyclonal immunoglobulins and their inclusion in the Brouet classification as type II-III variant has been proposed [53,54]. The IgM micro-heterogeneity, identified in about 10% of cases, has been considered as possible intermediate state in the transition from type III to type II MC; this serological condition and the transformation from polyclonal to oligoclonal and finally monoclonal IgM-RF fraction, reflects the continuous B cell clones expansion [54].

The process of cryoprecipitation is not entirely understood and probably differs between type I and II/III. In type I, the monoclonal component undergoes crystallization and aggregation, which is dependent on temperature and concentration [55]. Although the definition of cryoglobulins is precipitation at cold temperatures, this process can occur at room temperature at high cryoglobulin concentrations [46]: this fact probably explains why distal extremities (lower temperatures) and kidneys (increase in concentration as a result of ultrafiltration) are major sites of pathology. In type II/III cryoglobulinemia, cryoprecipitation takes place in the setting of immune complex formation between polyclonal IgG and IgM

with RF activity and complement fixation. Cryoprecipitation cannot be induced by the IgM or IgG components separately and requires specific antigen-avidity IgG molecules [56]: this aspect emphasizes the unique properties of cryoglobulins and explains the high incidence of HCV as a cause. Although the real mechanism of *in vitro* cryoprecipitation is rather obscure, non-specific Fc-Fc interactions might explain the self-aggregation of some Igs. Modifications of the primary structure of heavy and light chains are responsible, at least in part, for the different solubility of cryoglobulin and many studies on chemical analysis of cryoglobulins revealed a reduced concentration of sialic acid or a reduced amount of galactose in the Fc portion of the Ig. Furthermore, the solubility of proteins also depends on various factors such as concentration and temperature. Decreasing the temperature causes changes in the steric conformation of the molecules exposing non-polar residues resulting in solubility loss. Another factor is the pH of the solution, which may affect secondary and tertiary structures of immunoglobulins [57].

In the presence of cryoglobulins in serum, the diagnosis of cryoglobulinemia is based on typical clinical manifestations; false negative results may occur as a consequence of improper sample handling. Samples should be transferred and centrifuged at 37°C to avoid precipitation before serum extraction. In type I, precipitation at 1 to 4 °C usually occurs within hours; samples should be stored for 7 days because precipitation can be delayed in the mixed types [58]. When cryoglobulins are detected, the measurement of the cryocrit, defined as the relative volume of the precipitate as a percentage of the total serum volume, should be reported. The correlation between the cryocrit and disease manifestation is poor [59,60], although higher cryocrit has been reported to increase the likelihood of symptomatic disease [52]; in a few studies the cryocrit was prognostic but overall, its use should be reserved for diagnosis because there is a poor correlation between the cryocrit and the response to treatment [58]. In normal human sera small amounts of cryoprecipitable immunoglobulins are frequently detected, reflecting specific molecular interactions between Ig molecules [61]. On the contrary, the

cryoprecipitation process occurring in cryoglobulinemia is caused by intrinsic characteristics of both monoclonal and polyclonal IgM components and can also be caused by interaction among single components of the cryoprecipitate.

1.4 HCV-associated mixed cryoglobulinemia

MC is the most frequent and well known extrahepatic manifestation developing during HCV infection; it is an autoimmune and non-malignant LPD characterized by the expansion of monoclonal IgM^+CD27^+ B cells producing a polyreactive natural antibody (Ab) endowed with RF activity. Upon binding of these monoclonal IgMs to the endogenous polyclonal IgGs, the resulting immune complexes (cryoglobulins) may precipitate and settle on the small vessels (predominantly capillaries, venules or arterioles) causing vascular inflammation within the skin, joints, peripheral nervous system and/or kidneys [62].

The major clinical manifestations of MC syndrome include palpable purpura, renal disease, arthralgias or arthritis, nonspecific systemic symptoms including weakness, peripheral neuropathy and hypocomplementemia (with the fall in C4 levels often being most prominent) [60,63,64]. The triad of purpura, arthralgia and weakness, named "Meltzer's triad", is well known but is seen in only one third of patients [65]. Purpura, which can be transient or persistent, is the most common manifestation: it's seen in up to 90% of patients and is a key feature of the diagnosis [56]. Patients may present with various combinations of these features and different manifestations may predominate at different times in an individual patient. The cryoprecipitate also contains most of the viral antigens, their corresponding antibodies and HCV-RNA whose concentration is from 10 to 1000 fold greater than in the supernatant; this fact support the primary role of HCV infection in cryoglobulinemia [66]. Several other findings suggested a close association between HCV and MC: the presence of HCV-RNA sequence in serum, bone marrow cells and PBMCs [67], the presence of anti-HCV antibodies, HCV antigens and viral RNA in the damaged tissues [68]. It is not yet clear why HCV induces the production of

MC in some patients but not in others, indicating that other factors may affect the development of cryoglobulinemia.

The production of IgM RF molecules is one of the most important events for the emergence of cryo-precipitable immune-complexes [69]. In normal conditions, RFs are molecules involved in the presentation of antigens to T cells within an immunocomplex. A postulated hypothesis for the pathogenesis of HCV-related MC is that HCV initially stimulates the production of IgM without RF activity. The persistent antigenic stimulation of complexes composed by polyclonal IgG/HCV and HCV/very low density lipoprotein may induce somatic mutations causing the acquisition of RF activity [57].

MC is frequently associated with the production of autoantibodies [70] including antinuclear antibodies (ANA), SSA/Ro and SSB/La [71], anti-cardiolipin and anti-neutrophil cytoplasmic antibodies (ANCA) [72]. The mechanism by which HCV induces non-organ-specific autoantibodies is unclear and may involve the enhancement of B-cell activation through the binding of the E2 envelope glycoprotein to the CD81 activatory co-receptor [73] and the activation of the innate Toll-like receptor (TLR) 7 by viral RNA [74].

An alarming complication of MC is represented by its evolution to B-NHL. Notably, the presence of MC in HCV positive patients may increase the risk to develop NHL; indeed, an Italian multicenter study showed an over 35-fold increased risk to develop NHL for HCV positive patients with symptomatic MC compared with the general population [75] and consecutively, approximately 8-10% of patients with HCV-MC progress to overt NHL. Even after eradication, HCV-MC patients may remain at high risk to develop a B-NHL [76]. The B-NHL subtypes most frequently described as being associated with HCV are marginal zone lymphomas (MZL), in particular splenic marginal zone lymphomas (SMZL), lymphoplasmacytic lymphoma (LPL), and diffuse large B-cell lymphoma (DLBCL) [77].

1.5 V_H1-69-expressing B cells in mixed cryoglobulinemia

The monoclonal RF of approximately 80% of patients with HCV-associated MC express a cross-reactive idiotype, called WA [78], which is commonly but not exclusively associated with the V_H1-69 heavy chain variable gene and with the V_K3-20 light chain variable gene [33,35]. It has been shown that V_H1-69-encoded stereotyped idiotypes are frequently expressed by chronic lymphocytic leukemia (CLL) cells and by some other human lymphoid malignancies, suggesting that these tumors arise as a consequence of the antigenic pressure by common epitopes [79]. A useful marker for investigating, by flow cytometry, MC monoclonal B cells putatively expressing the WA idiotype is represented by the monoclonal antibody G6, directed to the V_H1-69 protein product [33]; indeed, increased proportions of G6-positive B cells were found in 30% of patients with HCV-associated MC [80]. Studies of hybridomas and of cloned immunoglobulin genes derived from patients with HCV infection [81,82] suggested that, in addition to RF activity, the V_H1-69-encoded idiotype recognizes the E2 envelope glycoprotein of HCV. Interestingly, germline V_H1-69 is also preferentially used by neutralizing antibodies against human influenza A virus [83] and HIV-1 [84], suggesting that the V_H1-69 variable gene plays a key role in primordial antiviral defense by encoding natural antibodies reactive with broadly shared, conserved structural elements important for infectivity. In patients with HCV-associated MC, V_H1-69-expressing B cells accumulate massively and, in some cases, replace the entire pool of circulating B cells. However, the absolute B cell numbers remain within normal limits and these patients have no signs of malignancy indicating that, although B cell homeostasis is subverted in MC, control mechanisms constrain the clonal expansion of V_H1-69$^+$ B cells, unless genetic changes take place [33]. The phenotype of the monoclonal V_H1-69-expressing B cells that accumulate in the blood of MC patients has been thoroughly investigated.

By making use of the V_H1-69-specific G6 and G8 antibodies, it was shown [33,85] that these cells are phenotypically heterogeneous and they can be distinguished into

two major populations [86,87]: one of them resembles typical $IgM^+IgD^+CD27^+CD21^+$ MZ B cells [88] and the other one is characterized by decreased expression of CD21, the CR2 complement receptor [89].

1.6 The complement receptor type II: CD21

Complement receptor type II (CR2, CD21) is a 145-kDa glycosylated single polypeptide chain consisting of a long extracellular domain of 15-16 short consensus repeat sequences, a transmembrane region and a short cytoplasmic domain [90,91]; it is expressed on B cells [92], where its levels vary depending on the maturation stage of the cell, follicular dendridic cells, thymocytes and a subset of peripheral T cells [93,94]. Ligands of CD21 include the complement fragments C3d, C3dg and iC3b, that are covalently bound to the target antigens [95,96] and, in addition, it has been proposed to bind ligands such as the Epstein-Barr Virus (EBV) envelope glycoprotein gp350/220, the low affinity Fc-receptor for IgE (FcεRII o CD23) and the cytokine interferon-alfa (IFN-α) [97].

On B cell surface, CD21 forms a complex together with CD19 and CD81 (TAPA-1) [98,99], which functions as a co-receptor to the BCR. Upon simultaneous binding of complement-tagged antigens by CD21 and the BCR, the threshold for B cell activation is reduced [95,100]. In this scenario, CD21 acts as a bridge between the co-receptor complex and the BCR resulting in a co-ligation that induces the phosphorylation of the cytoplasmic tail of CD19 by BCR-associated tyrosine kinases and amplifies downstream signaling (Fig. 3) [95,101].

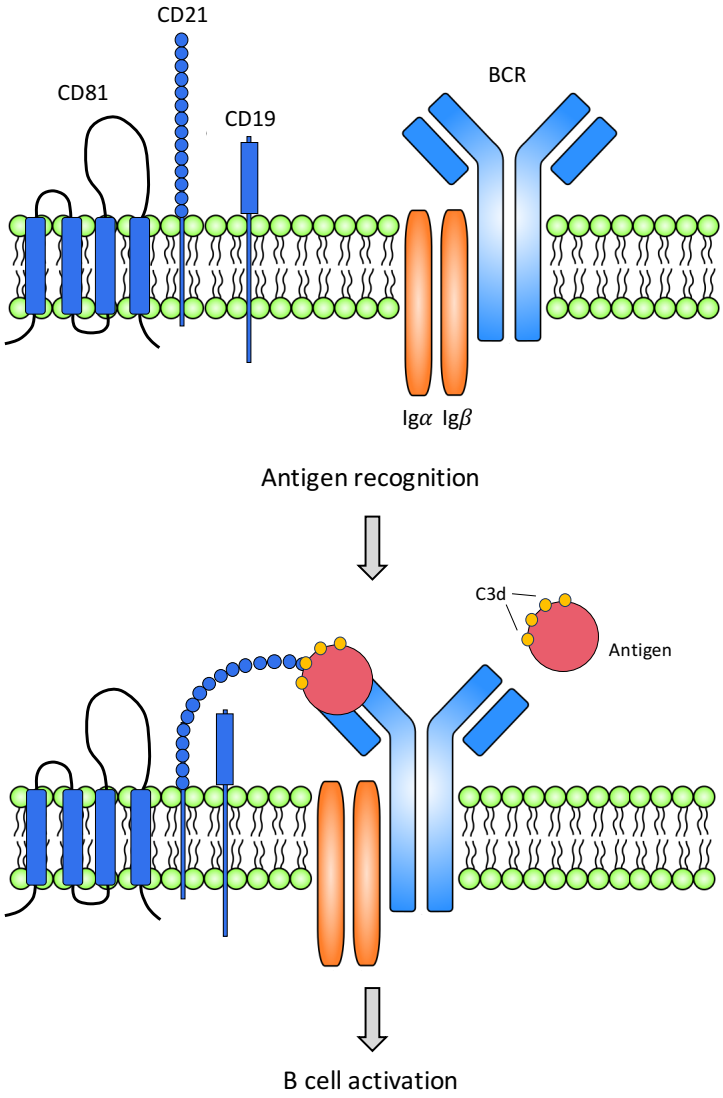


Figure 3. The BCR and its co-receptor

(Upper) The co-receptor complex, CD81, CD21 and CD19, and the membrane bound BCR together with its signal transduction molecules Igα and Igβ. (Lower) Upon simultaneous binding of complement-tagged antigens by CD21 and the BCR, the threshold required for B cell activation is reduced. Under these circumstances, CD21 acts as a bridge between the co-receptor complex and the BCR. The opsonizing complement fragments are C3d, C3dg or iC3b [112].

CD21 plays an important role in the selection for high-affinity B cells as well as the development and maintenance of B cell memory [102]. Although the BCR co-receptor function predominates, CD21 also mediates effects independent of the BCR including the induction of the transcription factor NF-kB, the production of interleukin-6 (IL-6) and the internalization of the antigen [95,103].

CD21 also exists in a soluble form, as the receptor can be shed from the plasma membrane by proteolytic cleavage in a short consensus repeat; indeed, it has been shown that CD21 is constantly shed from peripheral blood B lymphocytes and that its levels in blood are increased after cell activation. The amounts of soluble CD21 in blood are also increased in diseases such as common variable immunodeficiency (CVID), B-CLL and malignancies associated with EBV [104], whereas the levels are decreased in autoimmune diseases [105]. Increased frequencies of a B cell subset expressing low levels of CD21 ($CD21^{-/low}$ B cells) have been described in several diseases characterized by a chronic immune stimulation: HCV-associated MC [85], HIV infection [106], malaria [107], CVID [108], rheumatoid arthritis (RA) [109], systemic lupus erythematosus (SLE) [110] and Sjögren's syndrome [111].

In all these different conditions $CD21^{-/low}$ B cells could appear not to be the same as they express different BCR isotypes, and these can be either mutated or unmutated; they also differ in their expression of CD27 so much so in some disorders they were selected based on being $CD27^{+}$ or $CD27^{-}$. Despite these differences there are several similarities between $CD21^{-/low}$ B cells expanded in the various diseases mentioned above, such as the expression of the inhibitory receptor Fc receptor like 4 (FcRL4 or FcRH4), increased levels of CD11c, and a pattern of activation and homing receptors, the latter indicating migration to inflammatory sites [112].

More than a decade ago a $CD21^{-/low}$ B cell subset was found in human tonsils and described as a novel $CD27^{-}$ memory B cell subset that expressed FcRL4 [113,114]; these cells were defined as memory B cells because most were switched and their BCRs contained somatic hypermutations (SHM).

Although $CD21^{-/low}$ B cells have been extensively studied in several diseases, data concerning the presence of these cells in peripheral blood from healthy individuals are relatively poor [112].

1.7 $CD21^{low}$ B cells in mixed cryoglobulinemia and in other disorders

As previously reported, $CD21^{low}$ B cells of MC patients appear to be identical to those expanded in other immunological disorders with B cell hyperactivation, such as CVID, RA, and HIV infection. These cells, as well as those found in human tonsillar tissue, show signs of previous activation and proliferation, fail to proliferate in response to the stimulation of the BCR or of Toll-like receptor 9 (TLR9), are unable to flux calcium upon BCR cross-linking and are highly prone to die by apoptosis [80,86,87]. The same behavior has been observed even in $V_H1\text{-}69^+$ $IgM^+IgD^+CD27^+CD21^{high}$ MZ B cells, which therefore may be considered the precursors of their $CD21^{low}$ counterparts [85,89].

A striking common trait is the expression of CD11c, the integrin αx chain, typically a marker of dendritic cells. The function of CD11c in $CD21^{low}$ B cells is unknown but, interestingly, this receptor is also expressed in some splenic marginal zone lymphoma (SMZL) and in hairy cell leukemia [115]. Another peculiar marker of $CD21^{low}$ B cells is the inhibitory receptor FcRL4, first described in a subset of human tonsillar B cells with low CD21 expression [113]. Other inhibitory receptors of $CD21^{low}$ B cells are CD22, FcγRIIB (CD32b), CD72, CD85j, CD85k, leukocyte-associated Ig-like receptor-1 (LAIR-1) and sialic acid binding Ig-like lectin 6 (Siglec-6) [108,109,116]. The contribution of these inhibitory receptors, and particularly of FcRL4 and Siglec-6, to the dysfunction of $CD21^{low}$ B cells is supported by the partial recovery of the proliferative capacity and of the effector function upon silencing of these genes with siRNA [117]. A further peculiarity of $CD21^{low}$ B cells expanded in MC and in the other disorders is represented by the profile of trafficking receptors: indeed, it is possible to identify an increased expression of CD11c and CXCR3, which allow homing to sites of inflammation, and a reduced expression of CCR7,

CD62L, CXCR4 and CXCR5, which are required for migration to lymph nodes or to germinal centers [89,108,116]. Human $CD21^{low}$ B cells resemble, under some aspects, an expanded population recently identified in aged mice. These cells, described as $CD21^{low}$ $CD11c^{+}$ B cells and termed aged B cells (ABCs), are mostly autoreactive and develop more rapidly in female animals and in autoimmune-prone strains of mice [118,119]; interestingly, $CD21^{low}$ $CD11c^{+}$ ABCs show the T-box expressed in T cells (T-bet) transcriptional factors which is important for the control of viral infection. Signaling through TLR7 is crucial for ABCs development; moreover, like human $CD21^{low}$ B cells, ABCs respond poorly to BCR or CD40 triggering but rather they robustly respond to the stimulation of TLR7 or TLR9 [120]. Recently, $Tbet^{+}CD21^{low}CD11c^{+}$ B cells similar to murine ABCs were found significantly expanded in patients with chronic Hepatitis C or cirrhosis and it has been demonstrated that clearance of HCV infection reduces their overall frequency indicating a dependence on infection [121].

Microarray profiling studies of $CD21^{low}$ B cells obtained from patients with MC [86,87] or with CVID [108,109] or from normal human tonsillar tissue [114] revealed common gene expression signatures. Among the differentially expressed transcription factors, the most strikingly up-regulated in $CD21^{low}$ B cells are sex determining region Y (SRY)-box 5 (SOX5), runt-related transcription factor 2 (RUNX2), early growth response 2 (EGR2) and the zinc-finger homeobox 1B (ZFHX1B) that, interestingly, is deleted in HCV-associated high-grade diffuse large B cell lymphomas (DLBCL) [122]. Down-regulated genes include T cell leukemia/lymphoma1 (TCL1), which enhances B cell survival and is over-expressed in some B cell tumors, forkhead box 1 (FOXP1), a transcriptional repressor that is up-regulated in some DLBCL, and the IL-4 receptor. In $CD21^{low}$ B cells of MC patients it has been reported a striking up-regulation of Stra13, a basic helix-loop-helix transcription factor that acts as a key negative regulator of activation and cell cycle progression in B cells [81]. Stra13 was also over-expressed by $V_H1\text{-}69^{+}$ monoclonal B cells with high expression of CD21, while it was scarcely expressed

by $V_H1\text{-}69^+$ B cells from patients with MC-associated SMZL [80]. Microarray studies [86] revealed that the $CD21^{low}$ B cells of MC patients display increased transcripts of genes that favor apoptosis, such as galectin-1 (LGALS1), pyrin and HIN domain family-1 (PYHIN1), death-associated protein kinase 2 (DAPK2), myeloid nuclear differentiation antigen (MNDA) and Fas (CD95). High expression of these genes correlated with increased spontaneous [87] and culture-induced [86] apoptosis of $CD21^{low}$ B cells.

Although the differences in gene expression profiles of $CD21^{low}$ and $CD21^{high}$ $V_H1\text{-}69^+$ B cells have not been adequately investigated, a clear-cut difference between these cell populations is represented by the lack of inhibitory receptors (e.g. FCRL4, CD22, CD72 and CD95) on $CD21^{high}$ B cells, whereas a common feature is the overexpression of Stra13 [80].

1.8 The nature of BCR-mediated anergy

The ability of the adaptive immune system to provide protection against pathogens depends on a diverse repertoire of antigen receptors that allow recognition of a seemingly infinite range of foreign protein and carbohydrate antigens. Diversity is generated early in lymphocyte development by random rearrangement of immunoglobulin (Ig) genes; the random nature of this process leads inevitably to the generation of receptors that recognize self-antigens and that can be removed from the repertoire by a process of receptor editing [123] or clonal deletion [124]. However, despite these mechanisms of central tolerance silence B cells in the bone marrow, many self-reactive B cells escape to the periphery where they are silenced by anergy [125]. This last, defined as the failure to respond to BCR-mediated stimuli, is a component of normal B cell behavior [126]; it is a state of cellular lethargy resulting from the binding of the antigen by B cells (signal 1) in the absence of a significant $CD4^+$ T cell help (signal 2).

The concept of clonal anergy was first introduced in 1982 by Pike and Nossal, who observed that high doses of a monoclonal antibody with specificity for murine μ

chains (anti-Cμ), used as a "universal" B cell tolerogen because of its capacity to react with all emerging B lymphocytes regardless of specificity, prevented the development of normal numbers of B cells. By contrast, low concentrations of anti-Cμ resulted in normal B-cells expansion. However, unlike B cells developed in the absence of any anti-Cμ, these cells showed impaired both proliferation and antibody formation, and abrogated functional capacity: in other terms they were functionally anergic [127].

To explore the mechanism of anergy, it was necessary to develop transgenic mouse models in which this state of unresponsiveness can be induced in a controlled manner by creating a chronic interaction between antigen-specific B cells and antigen [126]. A favorite model, as well as the most widely studied, is represented by the hen egg lysozyme (HEL) double transgenic mouse, where persistent co-expression of a high affinity HEL-specific BCR and soluble HEL results in an anergic status. As a consequence, these mice fail to mount adaptive responses following immunization with exogenous HEL, and they exhibit a reduced capacity to generate antibody-secreting cells in response to the Toll-like receptor (TLR) agonists CpG containing DNA and lipopolysaccharide (LPS). In a parallel arsonate (Ars)-specific model, the transgenic BCR has modest affinity for the Ars hapten, yet cross-reacts weakly with endogenous antigen (most likely single-stranded DNA) with a similar anergic outcome [128].

It has been demonstrated that anergy is due to the continuous receptor occupancy by self-antigen, while antigen removal results in a rapid recovery of antigen receptor signaling [129]. Two key signaling features distinguish anergic cells from other types of B lymphocytes: reduced amplitude of phosphor-protein and $Ca2^{+}$ responses to antigen receptor ligation and elevated constitutive protein phosphorylation in intact cells. These signaling properties have been found both in mice and humans [126,130].

Although the pathways of positive BCR signaling in normal B cells which activate the signalosome and downstream pathways linked to proliferation, survival and

migration have been widely described [131], much less is known about signaling pathways operating in anergic cells. Chronic antigen signaling appears to lead to a shift in the balance between BCR-activation pathways and opposing inhibitory pathways but the means of achieving this are unclear [128]. As known, BCR has to be phosphorylated on the immunoreceptor tyrosine-based activatory motifs (ITAM) within Ig-α/Ig-β to initiate signal transduction. When the receptor is not engaged, ITAM are unphosphorylated and the BCR is off. Acute occupancy of receptors leads to bi-phosphorylation and consequent engagement of Syk tyrosine kinase and downstream effectors and adaptors (Fig. 4A). On the other hand, chronic BCR activation leads to ITAM mono-phosphorylation resulting in better activation of Lyn rather than Syk (containing dual SH2 domains, its activation requires two pY binding sites) and consequently in the stimulation of downstream inhibitory signaling pathways (Fig. 4B) [132]. This latter situation is typical of the anergic B cells. As part of feedback control, ITAM mono-phosphorylation results in the inhibition of signaling via phosphatases: SHIP1 activation plays a crucial role in anergy since it is constitutively phosphorylated in anergic cells. Other phosphatases, including SHP1 and PTEN, are also involved [133].

What causes the partial activation of downstream signaling responses, such as increased ERK phosphorylation and activation of NFAT, without increases in other kinases and transcription factors associated with positive responses (including AKT and NF-kB) [134] is unknown. ERK activity may play a role in the inhibition of signaling responses via TLR9, which can also be suppressed in anergic cells [135].

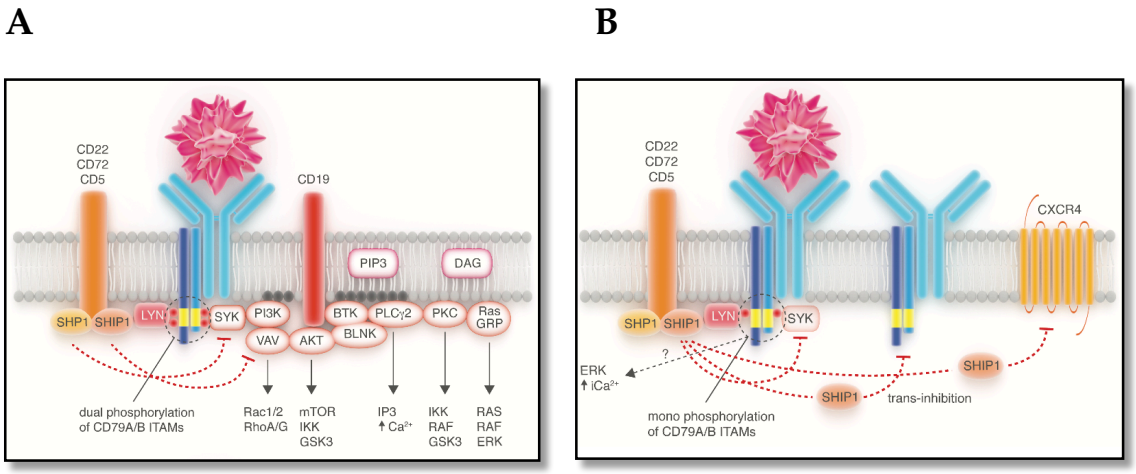


Figure 4. BCR signaling in naïve and anergic B cells

*Antigen triggers signaling that differs between naïve and chronically stimulated anergic cells. (**A**) In naïve B cells, antigen engagement triggers activation of Src-family kinases, including Lyn, which catalyzes the dual phosphorylation of immunoreceptor tyrosine-based activatory motifs within CD79 and CD79B. This results in recruitment and activation of Syk and triggers activation of downstream effectors, including protein kinases (Akt, Btk) and adaptors (BLNK), as well as lipid metabolism via lipid kinases (PI3K) and lipases PLCγ2 leading to the production of lipid metabolites, PIP3, DAG and IP3. These pathways are naturally inhibited by Lyn-dependent activation of both protein (SHP1) and lipid (SHIP1) phosphatases. (**B**) In anergic cells, chronic BCR engagement favors mono-phosphorylation of CD79A/B which results in weak Syk activation but efficient Lyn activation. Activation of phosphatases, especially SHIP1, further suppress Syk-dependent signaling by preventing accumulation of PIP3, and suppresses activation of BCRs and distinct receptors such as CXCR4. Anergic cells are often characterized by raised basal levels of iCa2+ and ERK phosphorylation [128].*

1.9 Functional exhaustion of $V_H1\text{-}69^+$ B cells in mixed cryoglobulinemia

Many features of $V_H1\text{-}69^+$ $CD21^{low}$ B cells of MC patients, and particularly the transcriptional profile obtained from gene expression studies, highlighted a condition of anergy, defective proliferation and reduced survival.

A decade ago, Moir and co-workers [116] defined the $CD21^{low}$ B cells of HIV-infected patients "exhausted" rather than anergic by analogy with virus-specific immune cells that have lost function because of the chronic nature of the viral infection. Indeed, $CD21^{low}$ B cells differ from classical anergic B cells under some aspects and especially because, unlike in most models of B cell anergy [126], they

are at least in some cases poised to secrete *in vitro* high amounts of immunoglobulins [108,113].

Several research groups have investigated whether, in HCV-associated MC, functional exhaustion is restricted to the $V_H1\text{-}69^+$ B cells with a $CD21^{low}$ phenotype or also concerns their $CD21^{high}$ counterpart: one of these groups showed that BCR-induced phosphorylation of ERK kinase was impaired not only in the $CD21^{low}$ but also in the $CD21^{high}$ $V_H1\text{-}69^+$ monoclonal B cells of MC patients [80,89]; in contrast with these findings, other two groups have shown that BCR-induced Ca^{2+} flux was defective in $CD21^{low}$ but not in $CD21^{high}$ B cells of HCV^+MC patients [86,87]. This discrepancy is most likely due to differences in the experimental design. Indeed, in the studies claiming that BCR signaling is defective only in $CD21^{low}$ B cells of MC patients, $V_H1\text{-}69^+$ B cells were not gated using the G6 antibody, since indirect staining with secondary anti-mouse Ig polyclonal antibody would have prevented experimental cross-linking of the BCR; therefore, the $CD21^{high}$ B cell populations examined were most likely contamined by normal polyclonal B cells. The other group [83] was also unable to gate the monoclonal B cells with the G6 antibody in BCR/ERK signaling experiments, but this problem was overcome by making use of negatively purified whole B cells from MC patients with a large predominance (70-98% of B cells) of $CD21^{high}$ $V_H1\text{-}69^+$ B cells, thus providing a clear evidence that also these cells, and not only the $CD21^{low}$ subset, have a defect in BCR signaling.

The $CD21^{low}$ but not the $CD21^{high}$ $V_H1\text{-}69^+$ B cells display also other features of anergy [86,87]. Comparing the capacity of FACS-sorted $CD27^+CD21^{low}$, $CD27^-CD21^{low}$, $CD27^+CD21^{high}$ and $CD27^-CD21^{high}$ populations of $V_H1\text{-}69^+$ B cells from MC patients to secrete IgM upon to co-stimulation with CD40 ligand (CD40L), IL2 and IL10, it was found that $V_H1\text{-}69^+$ $CD21^{low}$ B cells, irrespective of CD27 expression, secreted less IgM than their $CD21^{high}$ counterparts [86].

Interestingly, it was shown that $CD21^{low}$ but not $CD21^{high}$ B cells of MC patients failed to express activation markers and to proliferate in response to the stimulation with anti-IgM and CD40L, and that the $V_H1\text{-}69^+$ $CD21^{low}$ B cells responded normally

to the ligation of TLR9 with CpG [87]. However, unlike these findings, Visentini and co-workers [80,89] reported that the $CD21^{low}$ as well as the $CD21^{high}$ $V_H1\text{-}69^+$ B cells failed to proliferate in response to BCR or TLR9 ligation. The explanation of this difference is that in the first study the $CD21^{low}$ and the $CD21^{high}$ subsets seemingly included normally responsive polyclonal B cells, while in the second one [80,89] was exploited the staining with G6 antibody coupled to the CFSE dilution assay to unambiguously characterize the proliferative responses of $V_H1\text{-}69^+$ B cells to BCR or TLR9 stimulation. In addition, the claim that $CD21^{low}$ B cells of
MC patients proliferate normally in response to TLR9 ligation [87] contrasts with previous data showing that the $CD21^{low}$ B cells from CVID or HIV-infected patients are poorly responsive to TLR9 stimulation [108,116]. Indeed, this statement [87] was not adequately supported by experimental data, since cells were stimulated with CpG in conjunction with anti-IgM and CD40L, a combination of stimuli known to override the unresponsiveness of $CD21^{low}$ B cells [80,116].

1.10 Evolution of antiviral therapy

The therapeutic approach to MC has conventionally been based on the use of steroids and immunosuppressive drugs; the primary goal of antiviral therapy is the achievement of a sustained virological response at 12 weeks (SVR12), which is recognized as the measure of treatment success and defined as undetectable HCV RNA in the blood at the end of treatment and again 12 weeks following treatment end. IFN-based therapy was introduced in 1987, before the identification of HCV as the major etiologic factor of MC [136,137]. For several years, IFN-α was the only agent available for the treatment of patients with chronic HCV infection; the addition of ribavirin (RBV), a guanosine analogue that produces broad-spectrum activity against several RNA and DNA viruses, to IFN-α increased the SVR rate to about 50 % [138,139]. In 2000, pegylated (p) IFN-α, in which inert polyethylene glycol is attached to conventional IFN-α, was introduce into the clinical practice: this modified form of the drug showed a longer half-life, markedly improved SVR

rates and offered the further advantage of weekly rather than thrice a week administration [140]. There are two types of pIFN- α which share over 95% amino acid sequence homology. As a result of their difference in size and structure, these two molecules have different *in vivo* and *in vitro* characteristics: while pIFN- α2a (40 kDa) has a longer half-life, is mainly catabolized in the liver and has active breakdown products, pIFN- α2b (12 kDa) is a smaller molecule, has a shorter half-life, and acts as a pro-drug depot by slowly releasing IFN.

The antiviral combination of pIFN-α 2a/2b plus ribavirin (RBV) was employed to obtain a SVR and, for many years, it has been considered the standard of care for the treatment of chronic HCV infection, capable of inducing SVR and clinical remission of vasculitis in about 78% of patients with MC [141,142].

A multicenter, open-label study showed viral response rates ranging from 36% to 64% according to viral genotype and a clinical response in 88% of the patients [143].

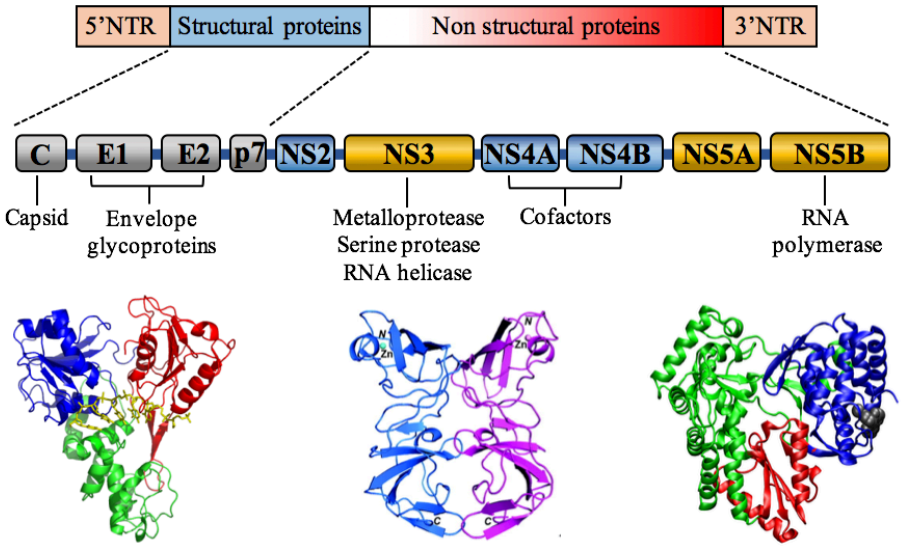
The association of rituximab (RTX), an anti-CD20 monoclonal antibody aimed at inducing an

effective B cell depletion, resulted in further benefits both in patients refractory to antiviral therapy [144,145] and in those previously untreated [146]; however, the efficacy of therapy in terms of viral clearance remained unsatisfactory and was burdened with remarkable side effects.

The use of first-generation protease inhibitors, such as boceprevir or telaprevir, associated with pIFN- α + RBV in a triple-combination therapeutic regimen resulted in significantly higher response rates in patients with chronic HCV infection without and with MC; nevertheless, serious adverse events were recorded in almost 50% of patients [147].

The introduction of all-oral, direct-acting antiviral agents (DAAs) has dramatically changed the treatment of chronic HCV and consequently the prognosis of these patients. Thus far, four classes of DAAs have been discovered (Fig. 5):

1) Protease inhibitors (asunaprevir, boceprevir, telaprevir, simeprevir, paritaprevir, grazoprevir, vaniprevir and voxilaprevir)
2) NS5A inhibitors (ledipasvir, daclatasvir, ombitasvir, elbasvir and velpatasvir)
3) Nucleotide polymerase inhibitors (sofosbuvir, dasabuvir)
4) Non-nucleotide polymerase inhibitors (deleobuvir, dasabuvir)



NS3	NS5A	NS5B
The NS3/4A serine protease	Multifunctional phosphoprotein, component of the HCV-RNA replication complex	RNA-dependent RNA polymerase
Protease Inhibitors	**NS5A Inhibitors**	**Polymerase Inhibitors**
Telaprevir	Daclatasvir	Nucleoside analogue
Boceprevir	Ledipasvir	GS-7977 (Sofosbuvir)
Simeprevir	ABT-267	Mericitabine
Faldaprevir	GS-5816	Non-nucleoside analogue
Asunaprevir	ACH-3102	BI-207127, ABT-333
ABT-450	Samatasvir	ABT-072, BMS-791325
MK-5172	MK-8742	Tegobuvir, Setrobuvir
Sovaprevir	GSK-23368	VX-222, Filibuvir

Figure 5. HCV genome and potential drug discovery targets
The HCV RNA genome serves as a template for viral replication and as a viral messenger RNA for viral production. It is translated into a polyprotein that is cleaved by proteases. All the HCV enzymes – NS2/3 and NS3-4A proteases, NS3 helicase and NS5B RNA dependent-RNA polymerase – are essential for HCV replication and are therefore potential drug discovery targets [148].

Protease inhibitors are potent drugs that can inhibit the enzymatic activity of NS3/NS4A proteases while presenting several potential limitations: indeed, they are highly specific and as the amino sequence of the NS3 protease domain differs significantly between HCV genotypes, they exhibit varying activities across genotypes. For instance, telaprevir is less effective in treatment-naïve subjects infected with genotypes other than genotype 1. Furthermore, as HCV has a high mutation replication rate, with a lack of proofreading, resistance is an issue for this class of drugs [148]. NS5A inhibitors might inhibit replication complex formation at the endoplasmic reticulum and sequester NS5A in lipid droplets to prevent virus formation and release [149]; without affecting the stability and the dimerization of NS5A, NS5A inhibitors not only block HCV RNA synthesis at the stage of membranous biogenesis [150], but also impair viral assembly by inhibiting the delivery of

HCV genomes to assembly sites [151]. In spite of their high potency, NS5A inhibitors slowly inhibit HCV RNA synthesis in comparison to HCV protease or polymerase inhibitors [152]. Nucleotide inhibitors bind to the catalytic site of NS5B and compete with incoming nucleoside triphosphates to interrupt the RNA synthesis. Non-nucleotide inhibitors bind to the allosteric binding pockets outside the NS5B catalytic site based on the non-competitive mechanism of action that inhibit the RNA synthesis [153].

All DAAs classes listed above are already available in Italy, except elbasvir, grazoprevir and velpatasvir, for which approval is pending. Because the antiviral efficacy of individual DAAs usually depends on HCV genotype, genotypic subtype and disease severity (e.g., cirrhosis) and many DAAs are prone to induce HCV resistance when used alone, combination regimens including two, three or even four DAAs, without pIFN-α, have been developed and have become the new standard of care for HCV. These drugs have been shown to be capable of eradicating the infection in over 90% of patients with chronic HCV, with limited variations related to viral genotype [154]. An additional advantage of DAAs is the possibility

of avoiding IFN-related side effects. However, owing to the high costs of these drugs, it has been necessary to fix priority criteria among patients to be treated. Both the American and the European associations for the study of liver diseases, as well as the Italian Medicines Agency (AIFA), have established that extrahepatic manifestations of HCV infection, such as cryoglobulinemic vasculitis and lymphoproliferative disorders, should be recognized as priority indications for treatment with DAAs [155,156].

So far, only a few papers have been published on the efficacy and safety of the use of DAAs in patients with MC. In particular, Gragnani et al. [157], in a prospective double-centre (Florence/Rome) study of a cohort of 44 consecutive patients with HCV-associated MC, 2 of whom had evolved into an indolent NHL with monoclonal B cell lymphocytosis, have shown that following guideline-tailored therapy with DAAs it is possible to achieve, in these patients, very high virological and clinical response rates: indeed, a striking reduction in the mean cryocrit value was revealed at SVR12, and an even greater reduction was observed for sustained virological response 24 weeks after therapy completion (SVR24); a partial vasculitis response and a roughly 50% reduction of cryocrit were detected in the two patients with lymphoma, and adverse events were rather frequent but usually mild [157].

Chapter 2

OBJECTIVE OF THE STUDY

Anergy is one of the mechanisms that the immune system adopts to silence autoreactive B lymphocytes upon low-affinity recognition of self-antigens (Ag); in this condition, self-reactive B cells persist in the periphery yet remain unresponsive to immunogen. The molecular mechanisms of B cell anergy have been dissected in several transgenic mouse models [126]. A relevant issue was to understand whether the anergic status was induced after a transient exposure to a self-Ag and "remembered" or if it required continuous Ag receptor occupancy and signal transduction.

Just over 10 years ago, Gauld and co-workers [129] explored this issue using an immunoglobulin-transgenic mouse model in which B cells were specific for the hapten arsonate (Ars) yet cross-reacted with a self-Ag that induced anergy *in vivo*. Their findings have clearly shown that continuous binding of Ag and subsequent receptor signaling are essential for the maintenance of the anergic status, since many features of B cell anergy can be rapidly reversed after dissociation of self-Ag using hapten competition, allowing these cells to recover Ag responsiveness.

An elegant study conducted by Goodnow and co-workers has shown that in mice made tolerant by transgenic hen egg lysozyme (HEL) [135], ERK kinase is constitutively activated and provides a tolerogenic signal that inhibits TLR9-induced plasma cell differentiation; interestingly, after disengagement of the BCR from the self-HEL the ERK phosphorylation was reduced.

IgM^+CD27^+ B cells clonally expanded in patients with HCV-associated MC display peculiar phenotypic and functional features. In particular, these cells, chronically stimulated by HCV, display both features of anergy, induced by continual engagement of the BCR, such as high expression of the active phosphorylated form of the ERK kinase (pERK) and reduced lifespan, and of virus-specific exhaustion

such as a $CD21^{low}$ phenotype and defective response to the stimulation of the BCR and of TLR9. Identical $CD21^{low}$ B cells are also expanded in patients with CVID and in HIV-infected individuals; in these cells, the high constitutive expression of pERK, together with reduced calcium flux and propensity for apoptosis, makes them closely resembling murine B cells made anergic by continual BCR engagement by antigen.

Usually MC regresses after eradication of HCV with IFN: indeed, in a study of 2012, Visentini and co-workers demonstrated that clinical signs of vasculitis regressed and the cryocrit values decreased significantly in all patients with sustained virological response (SVR) studied. In addition, they observed that HCV-RNA in serum became negative two to five months later the beginning of IFN-based therapy and both the absolute number and the percentage of $V_H1\text{-}69^+$ B cells declined in patients with SVR but not in virological non-responders [161].
However, the immunomodulatory and anti-proliferative activities typical of IFN might contribute to the observed effects.
In this work, I exploited the newly available DAAs, which rapidly suppress HCV viremia in HCV^+MC patients and lack the immunomodulatory and anti-proliferative properties typical of IFN, to find analogies with the mouse model and to untangle the effects of BCR disengagement in a human model of virus-driven anergy and exhaustion.

Chapter 3

MATERIALS AND METHODS

3.1 Mixed Cryoglobulinemia patients

Twenty-four patients (8 males/16 females; median age 65 y, range 38-82 y) with HCV-associated MC vasculitis were treated with DAA combinations tailored according to current guidelines as previously described (Table 1) [157]. Thirteen patients were infected with HCV genotype (GT) 1, 8 with GT 2, 2 with GT 3 and 1 with GT 4. Sixteen patients had chronic hepatitis and 8 cirrhosis. All patients had negative HCV viremia at the end of treatment (EOT) and sustained virologic response (SVR) through the follow-up; 3 patients were followed-up only for 12 weeks (SVR12) and 20 for 24 weeks (SVR24) after completion of treatment. One patient was followed only up the EOT. Table 1 reports some of the patients' demographic, clinical and treatment characteristics.

Patient	Age/gender	HCV genotype	Liver disease	DAA therapy, weeks of treatment
1	70/f	1b	C	SOF+SIM, 12
2	58/m	2	CH	SOF+RBV, 12
3	77/f	1b	CH	SOF+LED, 12
4	69/m	1b	CH	SOF+LED, 12
5	57/m	4a	C	SOF+LED+RBV, 12
6	61/f	1b	CH	PAR/OMB/RTV+DAS, 12
7	58/f	1b	CH	SOF+LED+RBV, 12
8	38/f	3	C	SOF+DCV, 24
9	58/f	1a	CH	SOF+LED+RBV, 12
10	63/f	1b	CH	SOF+LED, 12
11	78/f	2	CH	SOF+DCV, 12
12	55/f	2	CH	SOF+RBV, 12
13	52/m	1b	CH	PAR/OMB/RTV+DAS, 12
14	68/m	2	C	SOF+RBV, 12
15	51/f	3	C	SOF+RBV, 24
16	76/f	1b	C	SOF+LED+RBV, 12
17	75/f	2	C	SOF+RBV, 12
18	72/m	2	CH	SOF+RBV, 12
19	78/m	1b	C	SOF+LED, 12
20	59/m	1a	CH	SOF+LED, 12
21	82/f	1b	CH	SOF+LED, 12
22	77/f	2	CH	SOF+RBV, 12
23	72/f	1a	CH	SOF+LED, 12
24	71/f	2	CH	SOF+RBV, 12

Table 1. Demographic, clinical and treatment characteristics of HCV$^+$ MC patients

Abbreviations: C, cirrhosis; CH, chronic hepatitis; DC, decompensated cirrhosis; DAS, Dasabuvir; DCV, Daclatasvir; LED, Ledipasvir; PAR/OMB/RTV, Paritaprevir, Ombitasvir, Ritonavir; RBV, Ribavirin; SIM, Simeprevir; SOF, Sofosvubir.

3.2 Cells and immunophenotyping

Peripheral blood mononuclear cells (PBMC) were obtained by density-gradient centrifugation. Immunophenotyping was done with combinations of fluorochrome-labeled monoclonal antibodies (Becton- Dickinson Biosciences). The G6 monoclonal antibody, which recognizes an epitope of the V_H1-69-encoded protein [158], was kindly provided by R. Jefferis (Birmingham, UK). Unlabeled G6 was counterstained with FITC- or PE-conjugated goat anti-mouse IgG (Becton-Dickinson Biosciences) using mouse IgG as control as previously described [33]. Flow cytometric analyses were done on a FACSCalibur instrument (Becton-Dickinson Biosciences) using the CellQuest (Becton-Dickinson Biosciences) and FlowJo (Tree Star, Ashland, OR) software.

The gating strategy for enumerating $CD21^{low}$ B cells is depicted in Fig. 1. B cells are co-stained with anti-CD20 peridinin chlorophyll (PerCP), anti-CD21 phycoerythrin (PE) and additional fluochrome-labeled antbodies as requested. A consensus gate defining B cells with high CD21 expression ($CD21^{high}$) was derived from the analysis of 11 healthy donors; setting the gate just below the cluster of $CD21^{high}$ B cells yielded percentages of B cells with low or no expression of CD21 ($CD21^{low}$) of 5.8 ± 2.5% (mean ± SD) in healthy donors and of 53 ± 23% in 24 patients with HCV^+ MC.

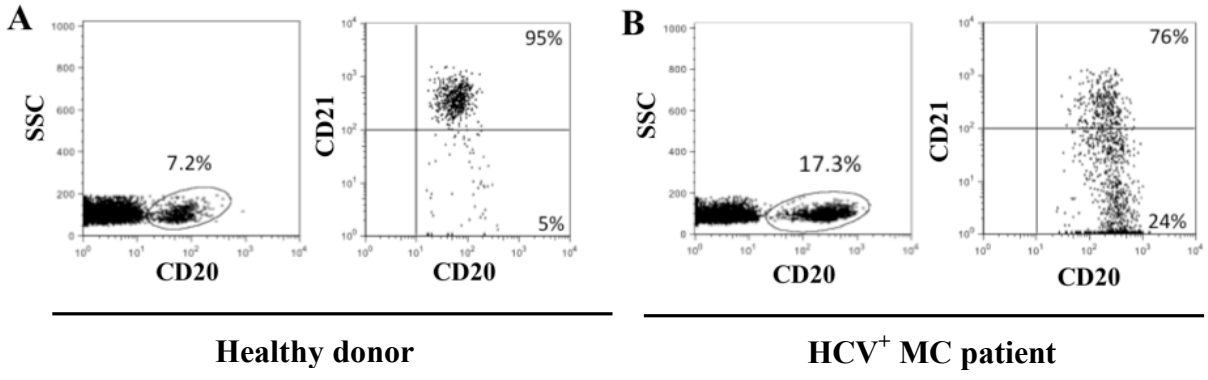


Figure 1. **Gating strategy for enumerating $CD21^{low}$ B cells**. *$CD20^+$ B cells from (A) one representative healthy donor and (B) one representative treatment-naïve patient with HCV^+ MC are electronically gated and percentages of $CD21^{low}$ B cells are calculated on the basis of a predefined consensus gate positioned at the 10^2 fluorescence channel.*

3.3 PhosFlow assay for pERK

The intracellular pERK content was measured by the BD Phos-Flow system as per manufacturer's Protocol 1 (Becton-Dickinson Biosciences). PBMCs ($1.5x10^6$ cells) were split in two vials, re-suspended in 100 µL of RPMI 1640 containing 5% fetal bovine serum (complete medium), and equilibrated at 37°C for at least 20 min. An equal volume of prewarmed complete medium, either alone (unstimulated control) or containing 10 µg/mL of F(ab')2 anti-human Ig (Jackson Immunoresearch Laboratories), was then added, and the cells were returned to 37◦C for 10 min. Cells were then fixed by the addition of 200 µL of prewarmed Phos-Flow Fix Buffer I for 10 min at 37° C, washed twice in PhosFlow Perm/Wash Buffer I, split in two vials, and stained either with anti-pERK1/2-Alexa 488 or with mouse IgG-Alexa 488 as control. Samples were simultaneously stained with fluochrome-conjugated mAbs to CD20, CD27, IgM, or with other mAbs as requested by the experimental design (e.g. CD21 or G6). The pERK-specific MFI was calculated by subtracting the MFI values obtained with control mouse IgG from those obtained with anti-pERK antibody. A representative experiment is shown in Fig. 2.

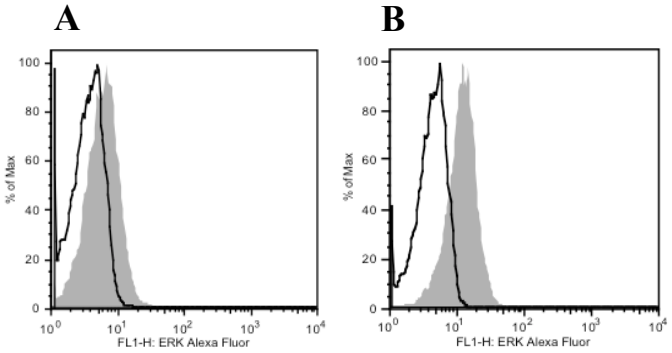


Figure 2. Phos-Flow assay of pERK expression in unstimulated B cells. *(A) healthy donor; (B) patient with HCV+ MC before DAA therapy.*
Open histograms denote staining with control antibody, and gray hystograms staining with anti-pERK.

3.4 ERK inhibition for apoptosis assays

B cells from HCV+ MC patients were enriched (>97%) by means of negative immunomagnetic sorting. For pharmacologic inhibition of ERK, cells were pre-incubated with 50 µmol/L U0126 (Calbiochem, San Diego, Calif) or dimethyl sulfoxide (vehicle) for 30 minutes at 37°C, before testing them for spontaneous apoptosis after 16 hours in vitro culture. After pre-treatment, the MEK/ERK

inhibitor U0126 was added to the culture medium and all subsequent steps for apoptosis assays were done in the presence of 50μmol/L U0126 or dimethyl sulfoxide.

3.5 Apoptosis assay

A total of 10^6 PBMC in 1 mL of RPMI containing 10% fetal bovine serum were incubated at 37°C for 16 hours in 96-well U-bottom plates ($2x10^5$ cells/well), and then washed, resuspended in phosphate-buffered saline with 10% fetal calf serum, and stained with Annexin V–phycoerythrin (Molecular Probes, Life Technologies), 7-amino- actinomycin (7AAD) (Sigma-Aldrich), anti-CD20 FITC and, in some experiments, G6 counterstained with PE-conjugated goat anti-mouse IgG. After electronic gating of $CD20^+$ B cells, early apoptotic B cells were identified as annexin $V^+/7AAD^-$, and late apoptotic cells as annexin $V^+/7AAD^+$ cells; the values reported in the Results and Discussion section refer to total apoptotic B cells (early plus late). A representative experiment is shown in Fig. 3.

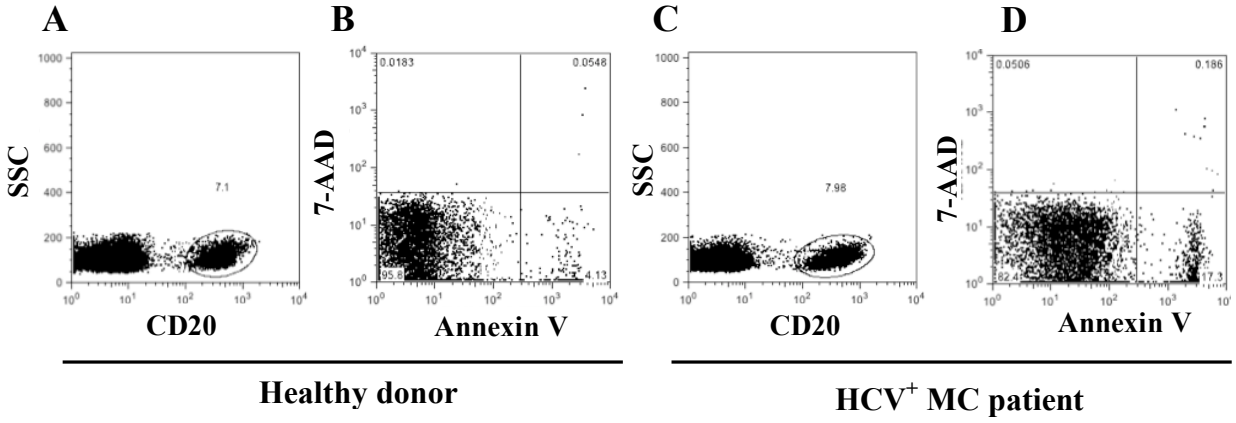


Figure 3. Spontaneous in vitro apoptosis of B cells. *After 16h in vitro incubation of PBMC in the absence of stimuli, cells were stained with anti-CD20, annexin V and 7-AAD. Electronically gated $CD20^+$ B cells from (A) a healthy donor and (C) a patient with HCV^+ MC were then analyzed for annexin V and 7-AAD staining. Early apoptotic B cells are identified as $V^+/7AAD^-$ and late apoptotic B cells as $V^+/7AAD^+$; total (early plus late) apoptotic B cells are (B) ~4% in the healthy donor's sample and (D) ~17% in the patient's sample.*

3.6 Cell proliferation assay

Cell proliferation was measured by the carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution assay [159]. PBMCs were labeled with CFSE (Invitrogen, Life Technologies) and cultured at 2×10^5 cells per well in 96-well U-bottom plates in the absence or presence of the TLR9 ligand CpG (2.5 μg/mL; Sigma Genosys). Cell proliferation was measured at day 5 of culture by flow cytometry. The number of cells that started dividing (precursor cohort) was calculated using the FlowJo software (Tree Star). Before flow cytometric analysis, cells were permeabilized (Permeabilizing-Solution 2; Becton-Dickinson Biosciences) and counterstained with antibodies to CD20, IgM and V_H1-69 (G6 antibody), or with other antibodies according to the experimental design. Cell proliferation was measured in electronically gated whole $CD20^+$ B cells or B cell subsets (e.g. $V_H1\text{-}69^+$ and $V_H1\text{-}69^-$ B cells). Plasmablasts were identified as $CD20^{low/neg}$ $cIgM^{high}$ cells.

3.7 Statistical analysis

Data were analyzed by the Mann–Whitney U test for unpaired two groups, by paired or unpaired t test, or by Wilcoxon signed rank test for paired two groups. Data were analyzed with StatView 5.0.1 software (SAS Institute, Cary, NC).

Chapter 4

RESULTS

4.1 Typical features of clonal B cells expanded in HCV-associated mixed cryoglobulinemia

Recent findings obtained in my laboratory have highlighted several typical features of clonal B cells expanded in HCV-associated MC [80]; the first step in my study was therefore to confirm the presence of these features even in patients enrolled.

Monoclonal B cells of approximately 80% of patients with HCV$^+$MC express a stereotyped idiotype which is commonly, but not exclusively, associated with the V_H1-69 heavy chain variable gene [33,35]. By making use of the V_H1-69-specific G6 antibody, I could identify, among the twenty-four patients screened, six patients with high proportions of circulating V_H1-69$^+$ B cells; flow cytometric analysis of a representative patient is shown in the figure 1A (II) and is compared to a healthy donor (HD) in which V_H1-69$^+$ B cells were absent [Fig. 1B(II)].

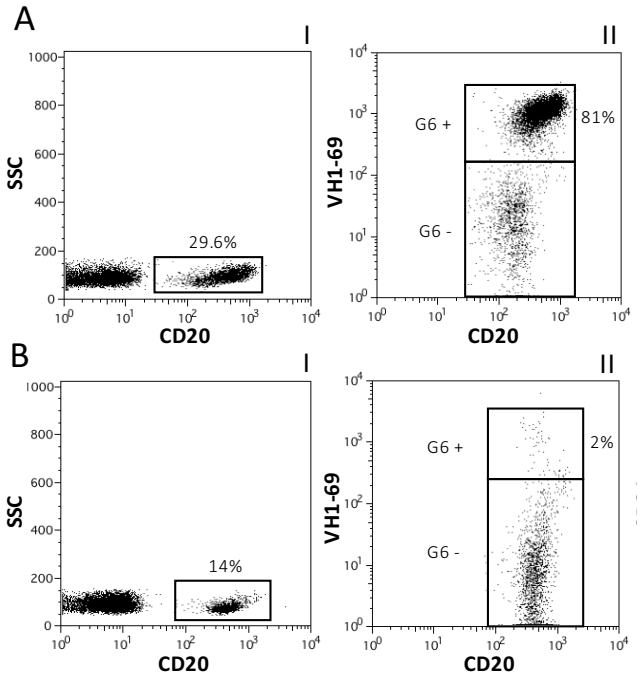


Figure 1. V_H1-69-expressing B cells in mixed cryoglobulinemia
Peripheral blood lymphocytes (PBL) from one representative patient with HCV$^+$MC (A) and one healthy donor (B) were co-stained with antibodies to CD20 and V_H1-69 (or with mouse IgG as control).
Dot plots show the electronic gating of V_H1-69$^+$ B cells (II), within total B cells (I), which are present in a high percentage in the HCV$^+$MC patient (A II) and absent in the healthy donor (B II).

These monoclonal B cells, that accumulate massively in the blood of patients, display both features of anergy, induced by continual engagement of the B cell receptor (BCR), and of virus-specific exhaustion. In mouse models it has been shown that B cells constantly exposed, *in vivo,* to antigen, become anergic because continuous engagement of the BCR delivers tolerogenic signaling through ERK kinase phosphorylation [129]; to find a correspondence with the murine model, and to confirm previous observations [80], I investigated whether patients' clonal B cells, which are chronically exposed to antigenic stimulation provided by HCV infection, display a similar pattern of constitutive and induced ERK phosphorylation.

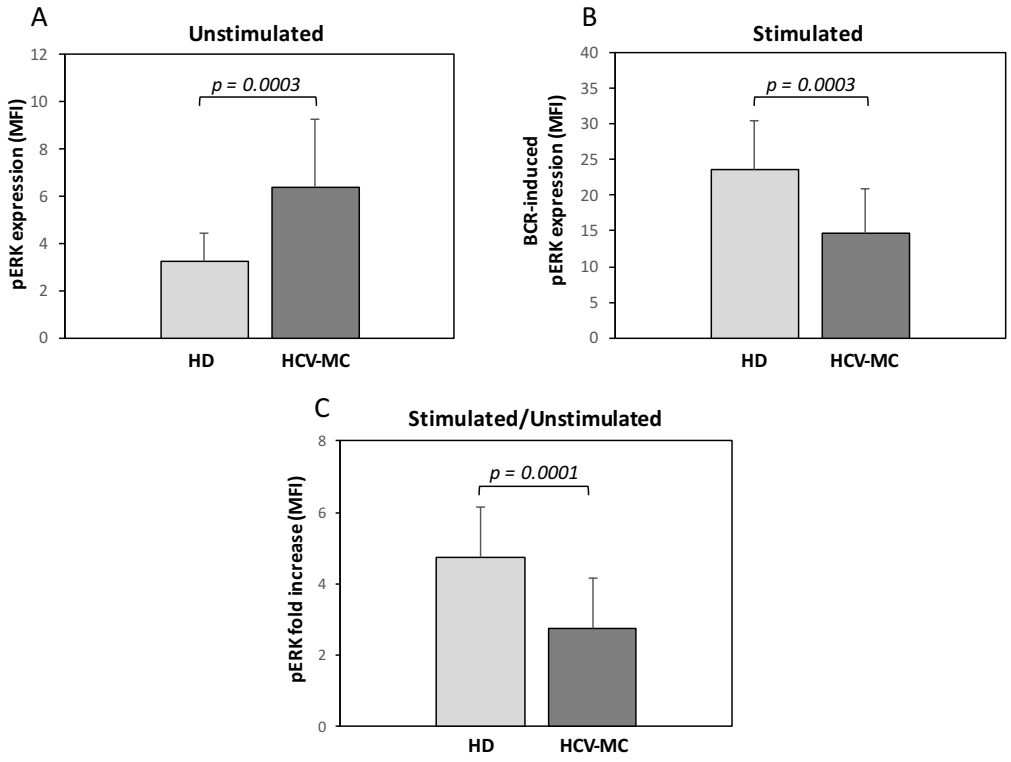


Figure 2. Constitutive and BCR-induced ERK phosphorylation

Peripheral blood mononuclear cells (PBMCs) from patients with HCV-associated mixed cryoglobulinemia (HCV-MC) or from healthy donors (HDs), left unstimulated or stimulated with anti-Ig, were co-stained with antibodies to CD20, IgM, and with either anti-pERK or control antibody. $CD20^+$ total B cells were analyzed for pERK expression. (A) Clonal B cells of HCV-MC patients display high constitutive pERK expression, compared to the expression levels observed in normal B cells of healthy donors (HD) (p=0.0003 using the Mann-Whitney test); both BCR-induced pERK expression (B) and pERK fold increase (C) were attenuated in HCV-MC patients compared to HDs (p=0.0003 and p=0.0001, respectively, using the Mann-Whitney test). Histograms denote mean values, expressed as Mean Fluorescence Intensity (MFI) and bars standard deviations.

In untreated patients, I noticed that clonal B cells expressed more basal pERK than normal B cells from the HDs (6.4±2.9% vs 3.2±1.2%, p=0.0003) [Fig. 2A], while BCR-induced ERK phosphorylation [Fig. 2B], as well as the rate of increase in pERK after BCR ligation (fold increase) [Fig. 2C] were reduced (p=0.0003 and p=0.0001 respectively). In particular, I observed that BCR cross-linking with anti-Ig induced a mean fivefold increase of pERK mean fluorescence intensity (MFI) in normal B cells and a mean threefold increase in HCV-MC B cells (4.73±1.43% vs 2.77±1.40%, p=0.0001) [Fig. 2C]. Uncoupling of constitutive and BCR-induced ERK activation was previously observed in $CD21^{low}$ B cells of HCV^{+} MC [82] and CVID patients [161].

Another typical feature of clonal B cells expanded in HCV^{+}MC patients is the reduced lifespan [86,87]. To assess this, I performed apoptosis experiments after 16 hours *in vitro* culture of B cells of untreated HCV^{+}MC patients, or HDs, without stimuli. The results obtained showed that clonal B cells of enrolled patients have a higher proneness to spontaneous apoptosis compared to HDs (15±11.2% vs 4.6±1.1%, p=0.0003) [Fig. 3A]. The figures 3B and 3C depict flow cytometric analyses of in vitro apoptosis without stimuli of B cells from a representative HD (3B) and a representative patient (3C).

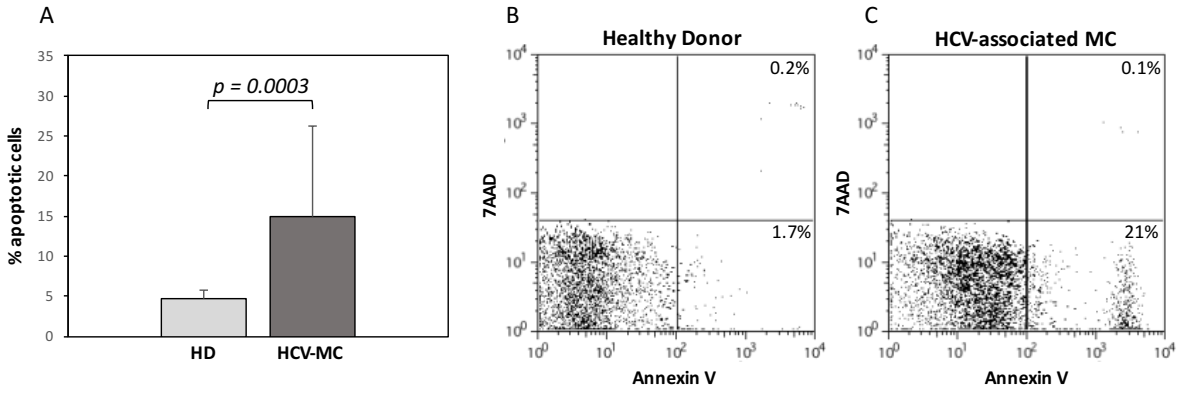


Figure 3. Spontaneous in vitro apoptosis of B cells

(A) Mean percentage of spontaneous in vitro apoptosis of B cells after 16h in vitro incubation of PBMCs without stimuli in patients with HCV⁺MC compared to healthy donors (bars denote standard deviations; statistical significance, indicated as p-value, was determined by the Mann-Whitney test); (B-C) electronically gated CD20⁺ B cells from (B) one representative healthy donor and (C) one representative patient with HCV⁺MC were analyzed for annexin V and 7-AAD staining. Early apoptotic B cells are identified as annexin V⁺/7AAD⁻ and late apoptotic B cells as annexin V⁺/7AAD⁺; total (eraly plus late) apoptotic B cells are (dot plot B) ~2% in the healthy donor's sample and (dot plot C) ~21% in the patient's sample.

In addition to these anergy-like features [131,137], HCV⁺MC clonal B cells also displayed the features of exhausted B cells [83,89,90]: indeed, most of them showed the typical $CD21^{low}$ phenotype (8.1± 4.1% HDs vs 63.9± 14.9% HCV-MC, p<0.0001) [Fig. 4A] and failed to proliferate in response to BCR or TLR9 stimulation (CpG: 37.9±7.8% HDs vs 18.0±17.1% HCV-MC, p=0.0150; CpG+Anti IgG-A-M: 72.4±7.1% HDs vs 27.5±20.3% HCV-MC, p=0.0001) [Fig. 4D].

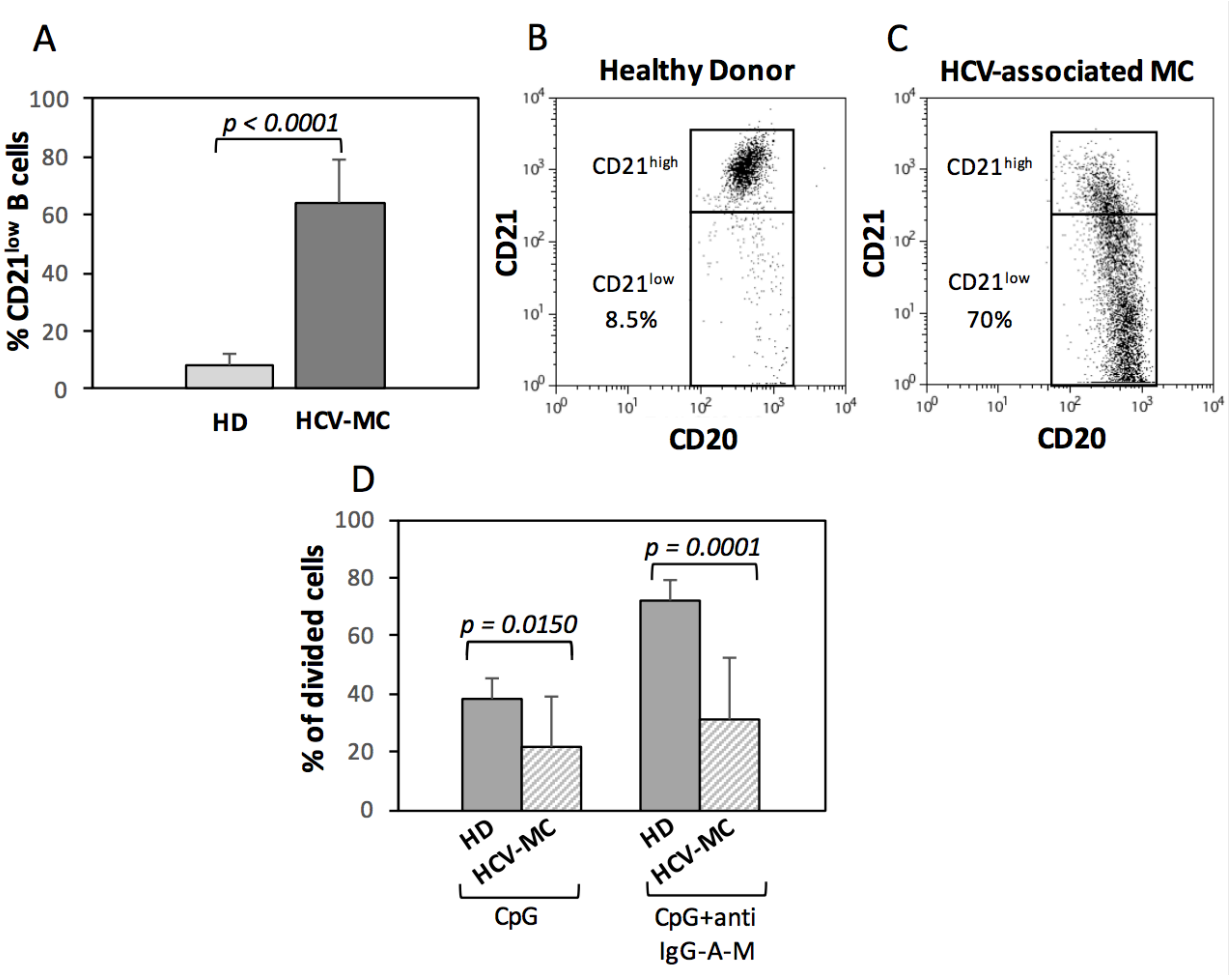


Figure 4. Phenotypic and functional features of clonal B cells of HCV-MC patients
(A) Mean percentage of CD21^{low} B cells in HCV^{+}MC patients compared to healthy donors (p<0.0001 using the Mann-Whitney test); (B-C) representative cytograms of one healthy donor (B), in which the proportion of CD21^{low} B cells is 8,5%, and one patient (C) with 68% of CD21^{low} B cells; (D) defective proliferative response in B cells of HCV^{+}MC patients, compared to HDs, following TLR9 and BCR stimulation (p=0.0150 and p=0.0001, respectively, using the Mann-Whitney test); histograms denote mean percentages and bars standard deviations.

To assess whether, in patients' clonal B cells, the high constitutive and attenuated BCR-induced pERK expression and the reduced lifespan were exclusive of CD21^{low} cells or even concerned their CD21^{high} counterparts, I evaluated these functional features in the two subsets.

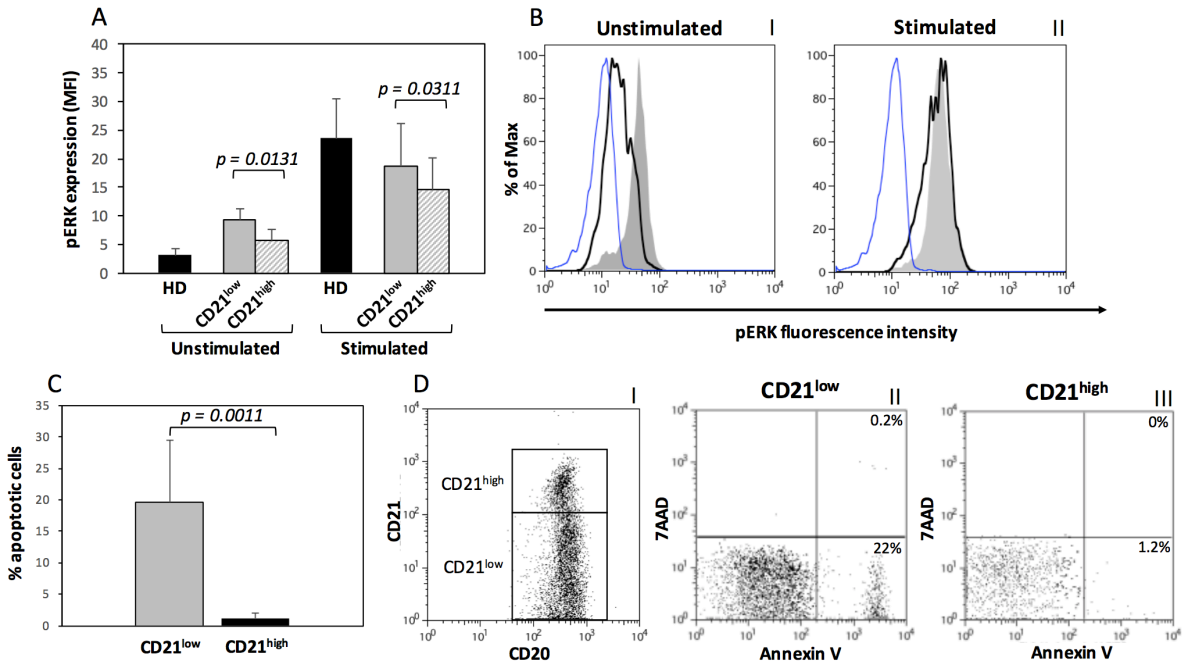


Figure 5. pERK expression and spontaneous in vitro apoptosis in CD21^{low} and CD21^{high} B cells from HCV^{+}MC patients

(A) Both CD21^{low} and CD21^{high} B cells of HCV-MC patients display high constitutive and attenuated BCR-induced ERK phosphorylation, compared to HDs, although in both cases the expression levels of pERK are higher in the CD21^{low} subset (p=0.0131 and p=0.0311 respectively, using the Mann-Whitney test; histograms denote mean values, expressed as Mean Fluorescence Intensity, MFI, and bars standard deviations);(B) analysis of unstimulated (I) and stimulated (II) B cells of a representative patient for pERK expression in CD21^{low} (gray-filled histograms) and CD21^{high} (open black histograms) subsets (open blue histograms denote staining with control antibody);(C) CD21^{low} B cells of HCV-MC patients are highly prone to die by spontaneous in vitro apoptosis compared to their CD21^{high} counterparts, in which the mean percentage of apoptotic cells is ~1.1±0.9 (vs 19.6±9.9%, p=0.0011 using the Mann-Whitney test; histograms denote mean percentages and bars standard deviations); (D) (I) electronically gated CD21^{low} and CD21^{high} B cells of a representative patient with HCV^{+}MC were analyzed for annexin V and 7-AAD staining: early apoptotic B cells are identified as annexin V^{+}/7AAD^{-} and late apoptotic B cells as annexin V^{+}/7AAD^{+}; total (eraly plus late) apoptotic B cells are (II) ~22% in the CD21^{low}subset and (III) ~1.2% in the CD21^{high} subset.

First of all, I noticed that $CD21^{high}$ B cells of screened patients showed a lower level of constitutive ERK phosphorylation, compared to their $CD21^{low}$ counterparts (9.3±2% $CD21^{low}$ vs 5.8±1.8% $CD21^{high}$, p=0.0131) [Fig. 5A, unstimulated], but still higher compared to that of HDs (3.2± 1.2%) [Fig. 2A]. BCR-induced ERK phosphorylation, as previously demonstrated [82,91], was impaired both in $CD21^{low}$ B cells (18.6±7.6%) and in $CD21^{high}$ B cells (14.5±5.5%), even though more accentuated in the latter (p=0.0311) [Fig. 5A, stimulated].

The analyses of spontaneous *in vitro* apoptosis revealed that the proneness to die is typical of $CD21^{low}$ B cells but not of their $CD21^{high}$ counterparts (19.6± 9.9% $CD21^{low}$ vs 1.1± 0.9% $CD21^{high}$, p=0.0011) [Fig. 5C-D].

4.2 Slow reduction of $V_H1\text{-}69^+$ and $CD21^{low}$ B cells after the withdrawal of chronic antigenic stimulation provided by HCV

Four weeks after the beginning of therapy with DAA, when all patients were negative for HCV RNA, the proportions of $CD21^{low}$ B cells were unchanged (Pre-therapy: 54.9±25% *vs* 4 weeks: 53.8±23%, p=NS) [Fig. 6A]. At the same time point, also the percentages of circulating V_H1-69-expressing B cells remained unmodified (Pre-therapy: 51.7±36.4% *vs* 4 weeks:49.7±36%, p=NS) [Fig. 6B].

Monitoring clonal B cells phenotype over time, I observed that the proportions of $CD21^{low}$ B cells declined steadily up to SVR24 [Fig. 6C], although they remained on average higher than in healthy donors (5.8±2.5%). However, the proportions of V_H1-69^+ B cells remained stable up to SVR24 in 3 out of 6 patients [Fig. 6D], suggesting that phenotypic changes occurred in clonal B cells after eradication of HCV as previously observed in HCV^+ MC patients treated with IFN [161].

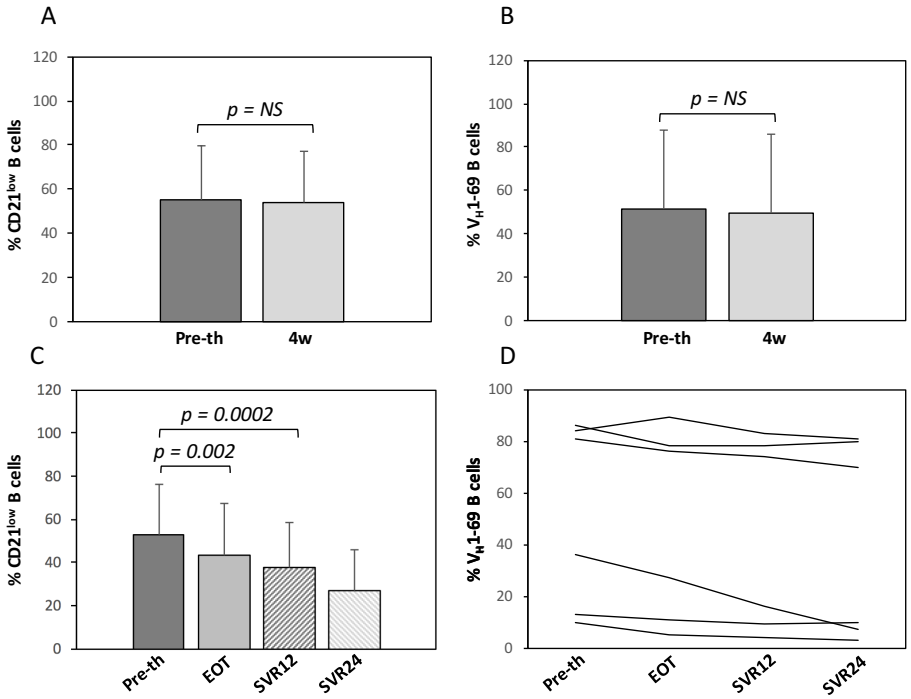


Figure 6. Changes in clonal B cells phenotype

No significant change in the mean percentage of CD21^{low} (A) and V$_H$1-69$^+$ (B) B cells four weeks after the beginning of DAA therapy (the p value is not significant in both cases, using the Wilcoxon signed rank test for paired two groups); (C) slow but steady decline of CD21^{low} B cells from baseline to SVR24; (D) changes in V$_H$1-69$^+$ B cell proportions over time; each line represents one patient followed from pre-therapy to SVR24. Histograms denote mean percentages and bars standard deviations; statistical significance, indicated as p-value, was determined by the Wilcoxon signed rank test for paired two groups.

In the figure 7, which depicts flow cytometric analyses of V$_H$1-69$^+$ CD21^{low} B cells in the 6 HCV$^+$MC patients, is shown a comparison between the frequency of CD21^{low} cells among V$_H$1-69-expressing B cells before DAA therapy and the frequency of the same cells 24 weeks after the end of therapy (SVR24). Peripheral Blood Lymphocytes (PBL) were stained with fluorochrome-labeled antibodies to CD20, CD21 and VH1-69; CD20$^+$ B cells were electronically gated and V$_H$1-69$^+$ cells were further gated for analysis of CD21 expression. Overall, data obtained in the 6 patients studied indicate that the percentages of V$_H$1-69$^+$ B cells among total B cells decreased slightly from pre-therapy (51.2±35.7%) to SVR24 (41.8±39.1) (p=0.066), while the percentages of CD21^{low} cells among V$_H$1-69-expressing B cells decreased more markedly, from 84.8±9.9% in the pre-therapy to 41.5±12% at SVR24 (p=0.043), indicating a reversion of the CD21^{low} phenotype.

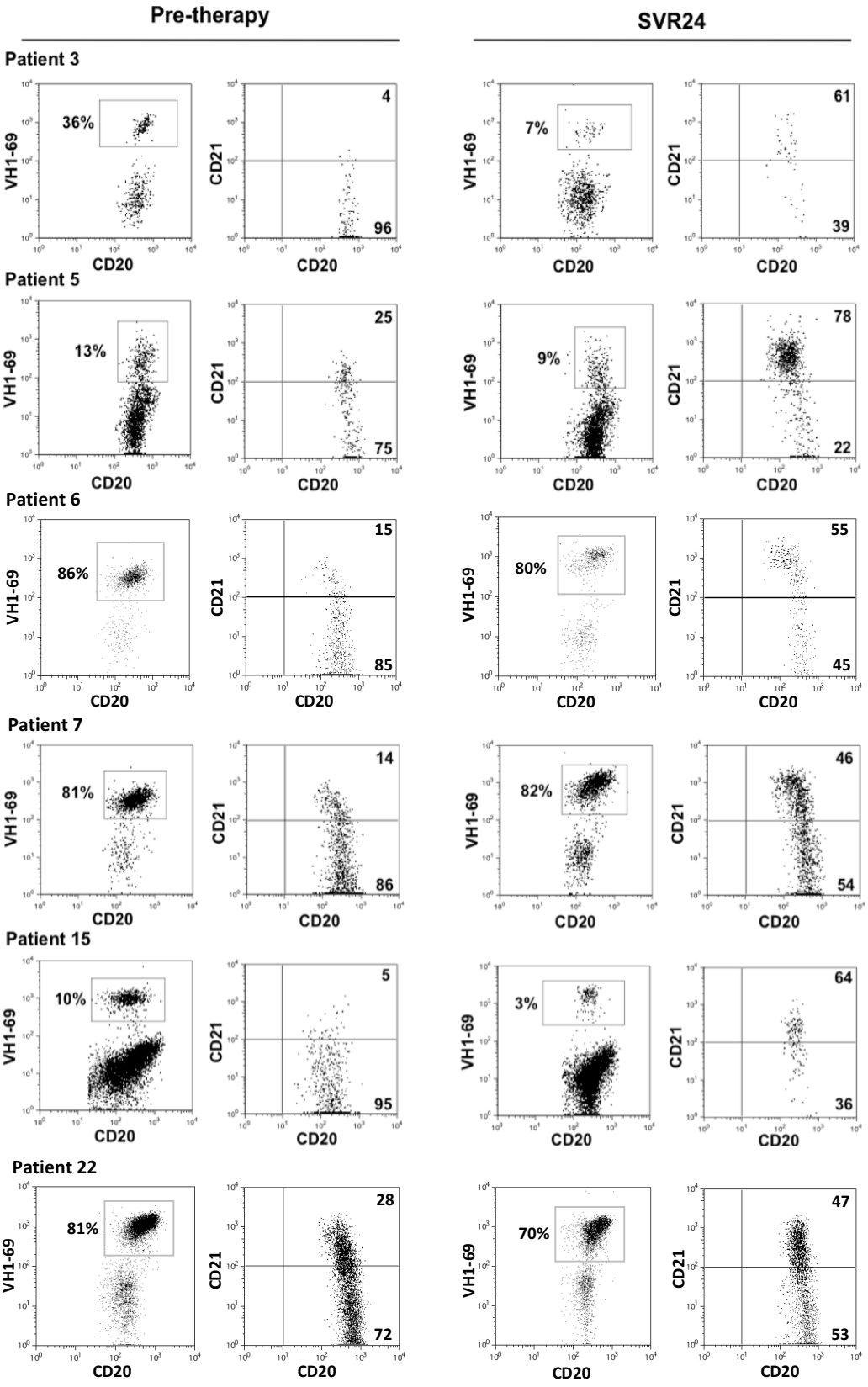


Figure 7. Frequency of CD21^{low} cells among V_H1-69-expressing clonal B cells decreases after antiviral therapy

4.3 "Rapid" resetting of the signaling machinery after clearance of HCV viremia

Another important observation made in the mouse model is that many features of anergic B cells, including high basal phosphorylation of ERK kinase, can be rapidly reversed after the dissociation of self-antigen from the BCR [129]. Similar to murine B cells made tolerant by constant BCR occupancy, clonal B cells expanded in HCV^{+}MC might be rendered anergic by continual stimulation due to chronic HCV infection; however, already after the first weeks of DAA therapy there's the clearance of HCV viremia, so that the chronic stimulus is missing. To further investigate the analogies with the mouse model, I analyzed ERK phosphorylation in some of the patients enrolled before and after the beginning of treatment.

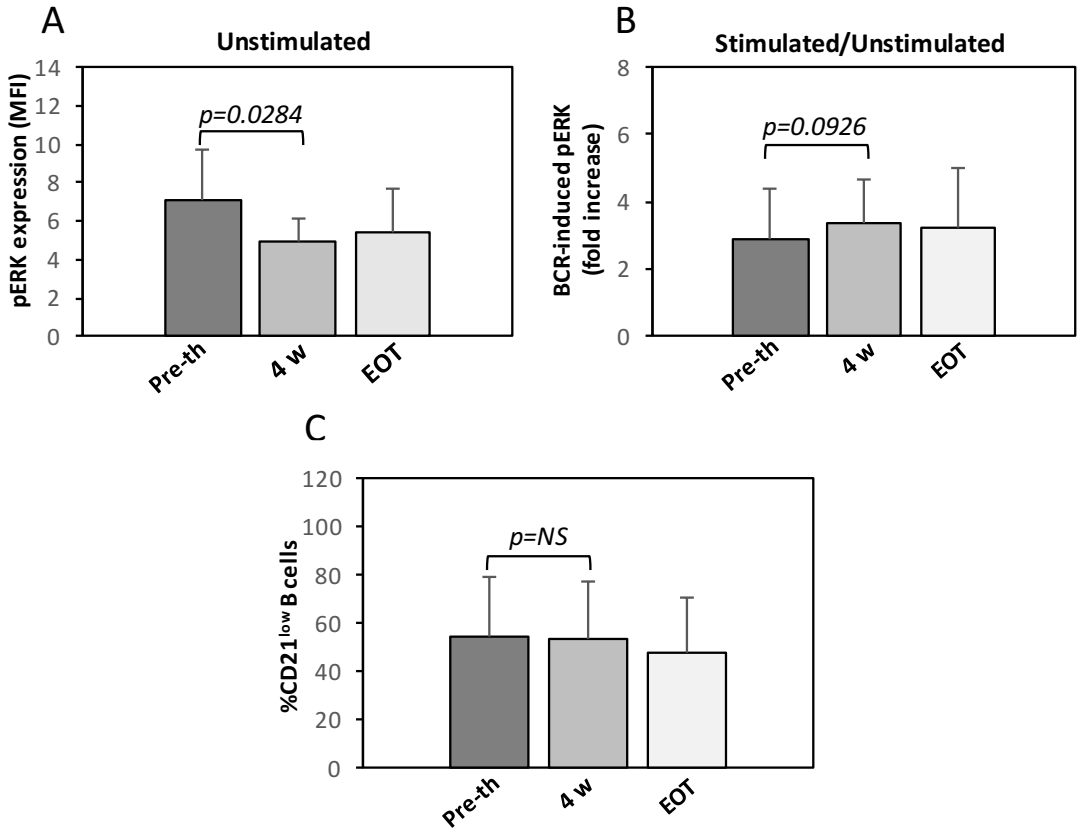


Figure 8. Early reduction of constitutive ERK phosphorylation in patients' total B cells
(A) Changes in constitutive pERK expression in clonal B cells of HCV^{+}MC patients; (B) rate of increase in pERK (fold increase) after BCR ligation; (C) mean proportions of CD21^{low} B cells from baseline to end of therapy (EOT). Histograms denote mean values, expressed as Mean Fluorescence Intensity (MFI) and bars standard deviations. Statistical significance, indicated as p-value, was determined by the Wilcoxon signed rank test for paired two groups

Four weeks after the beginning of DAA therapy, when all patients screened were negative for HCV viremia, constitutive ERK phosphorylation was significantly reduced compared to the pre-therapy levels (7.07±2.61 Pre-th vs 4.8±1.20 4w, p=0.0284) [Fig. 8A], although the rate of increase in pERK after BCR ligation (fold increase) increased only slightly (2.804±1.51 Pre-th vs 3.36±1.33 4w, p=0.0926) [Fig. 8B]. At the end of therapy (EOT) no further variation was observed, neither in constitutive ERK phosphorylation nor in BCR-induced ERK phosphorylation [Fig. 8 A-B].

The possibility that early normalization of constitutive ERK activation was due to the replacement of clonal B cells by normal B cells contrasts with the observation that the proportions of $CD21^{low}$ B cells in these patients were unchanged at week 4 of therapy (54.9±25% *vs* 53.8±23% pre-therapy, p=NS) [Fig. 8C].

To confirm the result obtained in total B cells of screened patients, I evaluated changes in pERK expression in the $CD21^{low}$ subset. Also in this case, four weeks after the beginning of DAA therapy there was a significant reduction of constitutive ERK phosphorylation both in $CD21^{low}$ (9.3±2.0% Pre-th vs 6.6±1.6 4w, p=0.0026) and in $CD21^{high}$ B cells (5.8±1.8% Pre-th vs 4.3±1.7% 4w, p=0.0277) [Fig. 9A]. At the end of treatment (EOT) no further significant reduction was observed in either of the two subsets [Fig. 9A]. BCR-induced ERK phosphorylation [Fig. 9B] was significantly increased at week four of therapy, compared to the pre-therapy levels, both in $CD21^{low}$ (18.6±7.6% Pre-th vs 22.6±7.7% 4w, p=0.0226) and in $CD21^{high}$ B cells (14.5±5.5% Pre-th vs 16.9±5.6% 4w, p=0.0185) [Fig. 9 B-C]. The relative increment, or fold increase, of pERK after BCR stimulation also significantly increased, from baseline to EOT, in both B cell subsets [Fig. 9C].

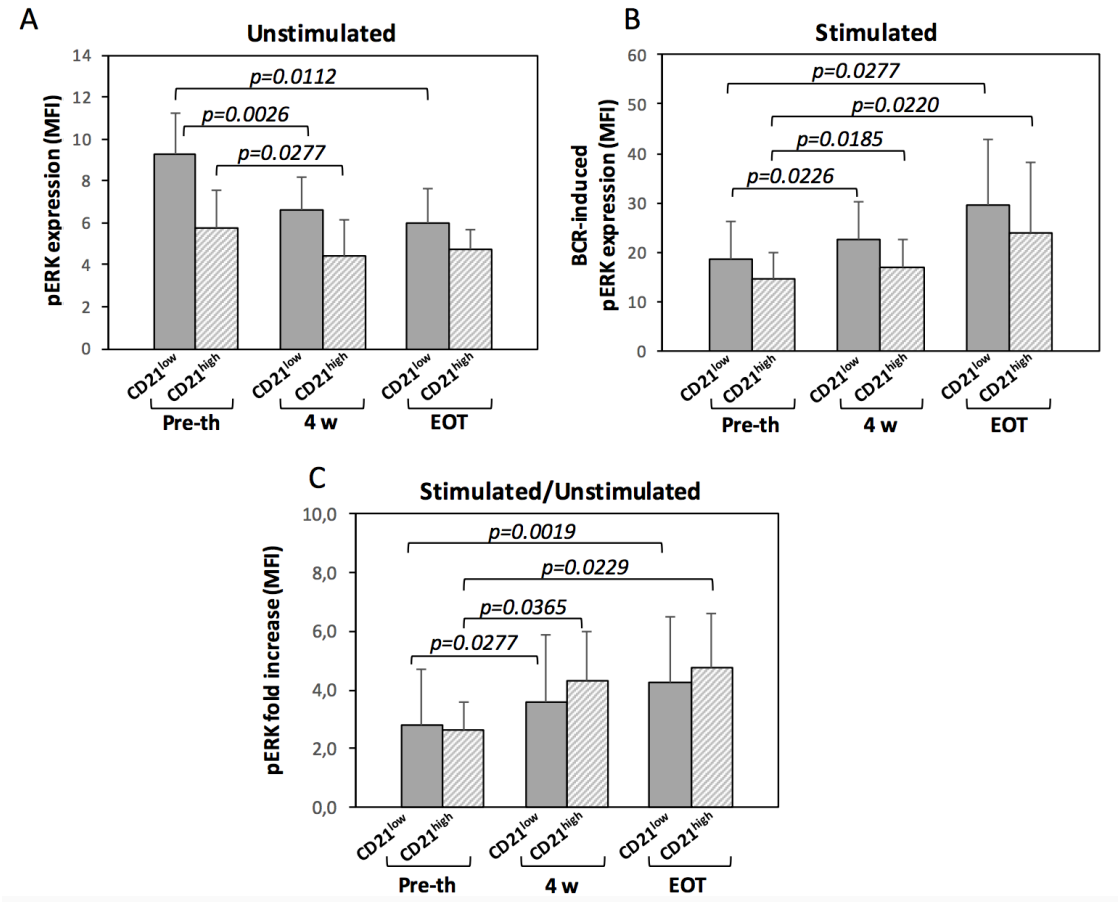


Figure 9. Changes in pERK expression in the CD21^{low} subset compared to their CD21^{high} counterparts. *Histograms denote mean values, expressed as Mean Fluorescence Intensity (MFI) and bars standard deviations. Statistical significance, indicated as p-value, was determined by paired t-test.*

4.4 Reduction of clonal B cells apoptosis after clearance of HCV viremia

As I mentioned before, clonal B cells of untreated HCV^{+}MC patients are highly prone to spontaneous in vitro apoptosis. Using a transgenic mouse model of B cell anergy, in which B cells were specific for the hapten arsonate (Ars) yet cross-reacted with a self-Ag that induced anergy *in vivo*, Gauld and co-workers [129] showed that the reduced lifespan of murine anergic B cells depended on chronic stimulation by self-antigen and that this effect could be reversed after the disengagement of the BCR. In order to evaluate whether even in clonal B cells of HCV^{+}MC patients the reduction in the lifespan depends on chronic stimulation and can be reversed by the disengagement of the BCR, I analyzed B cell apoptosis in some of the patients enrolled before and after treatment with DAA.

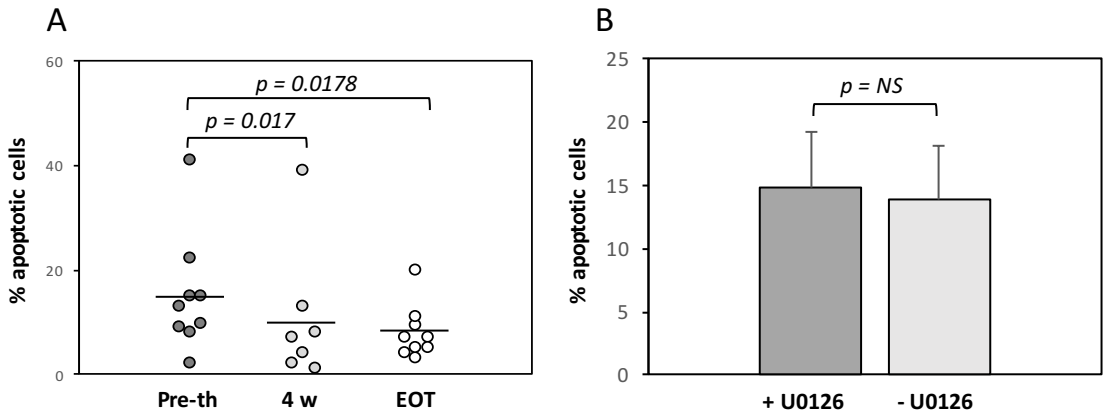


Figure 10. Reduction of B cell apoptosis after clearance of HCV viremia
(A) Reduction of spontaneous in vitro apoptosis of clonal B cells from HCV⁺MC patients after DAA therapy; each symbol represents a single patient studied once and bars denote the geometric means (statistical significance, indicated as p-value, was determined by the Wilcoxon signed rank test for paired to groups); (B) treatment with the MEK/ERK inhibitor U0126 failed to reduce spontaneous apoptosis of patients' B cells (histograms denote the mean percentages resulting from experiments in 4 different patients and bars denote standard deviations)

Four weeks after the beginning of DAA therapy, when all patients screened were negative for HCV RNA, the percentages of B cell apoptosis were significantly reduced (Pre-therapy: 15±11.2% vs 4w: 10±13.2%, p=0.017) [Fig. 10A]. At the end of treatment (EOT) there is a further reduction, albeit lower than the previous one (Pre-therapy: 15±11.2% vs EOT: 8±5.2%, p=0.0178) [Fig. 10A]. As well as for ERK signaling, even in this case the early normalization of spontaneous *in vitro* apoptosis cannot depend on the replacement of clonal B cells by normal B cells because the percentages of $CD21^{low}$ B cells, in these patients, were unchanged at week 4 of therapy (54.9±25% *vs* 53.8±23% pre-therapy) [Fig. 8C].

Although it is believed that reduced lifespan of murine anergic B cells is largely due to unfavorable competition for B-cell-activating factor (BAFF) [127], the rapid increase of lifespan after BCR disengagement suggests that chronic BCR signaling is sufficient to initiate apoptosis [132]. Thus, I investigated whether increased apoptosis of HCV⁺ MC B cells was related to ERK signaling; to this aim, I analyzed B cell apoptosis treating them with the U0126, a mitogen-activated protein kinase

(MEK) inhibitor that prevents MEK-dependent ERK phosphorylation. Treatment of MC B cells with U0126 failed to reduce spontaneous in vitro apoptosis, suggesting that ERK signaling is not directly involved in their pro-apoptotic pathway [Fig. 10B].

Even in this case, I confirmed the result obtained in total B cells evaluating spontaneous in vitro apoptosis in the $CD21^{low}$ subset. As indicated in the figure 11, the decrease in the proportions of apoptotic cells from pre-therapy to the end of treatment is confirmed also in $CD21^{low}$ B cells [Fig. 8].

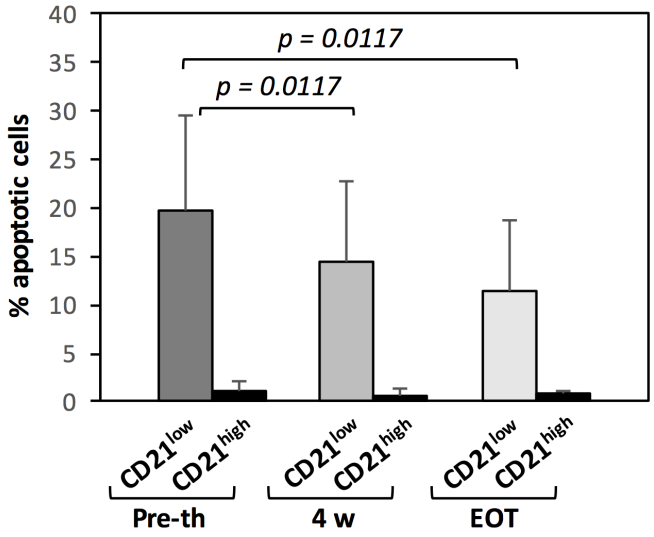


Figure 11. Proneness to die by apoptosis is typical of $CD21^{low}$ B cells but not of their $CD21^{high}$ counterparts

Reduction of spontaneous in vitro apoptosis in $CD21^{low}$ B cells, from baseline to end of therapy (EOT). Histograms denote mean percentages and bars standard deviations. Statistical significance, indicated as p-value, was obtained by the nonparametric Wilcoxon signed rank test for paired two groups.

4.5 Clonal B cell failure in recovering proliferative capacity after clearance of HCV viremia

As previously reported by my research team, both $CD21^{high}$ (MZ-like) and $CD21^{low}$ $V_H1\text{-}69^+$ B cells of untreated HCV^+ MC patients are functionally exhausted, since they fail to proliferate in response to the stimulation of TLR9 and BCR with their respective ligands [89]. After confirming this observation, already shown in the figure 4D of the paragraph 4.1, I investigated the possibility that $V_H1\text{-}69^+$ B cells could recover their proliferative capacity after removal of viral stimulus by DAA therapy. To this aim, as described in detail in *Materials and Methods*, CFSE labeled PBMCs were stained with the G6 antibody at the end of cultures. The strategy of analysis in a representative patient is illustrated in Figure 12. First of all, as previously shown in my laboratory [80], I observed that $V_H1\text{-}69^+$ B cells of untreated patients were unresponsive to CpG (Fig. 12A III) whereas autologous non-clonal $V_H1\text{-}69^-$ B cells proliferated robustly upon TLR9 stimulation (Fig. 12B II). At SVR24, when the viral stimulus was no longer present due DAA treatment, the situation doesn't change: indeed, the number of $V_H1\text{-}69^+$ B cells entering division (precursor cohort) was only 4% in the pre-therapy (Fig. 12A III) and just 8% twenty-four weeks after the end of therapy (SVR24) (Fig. 12B III).

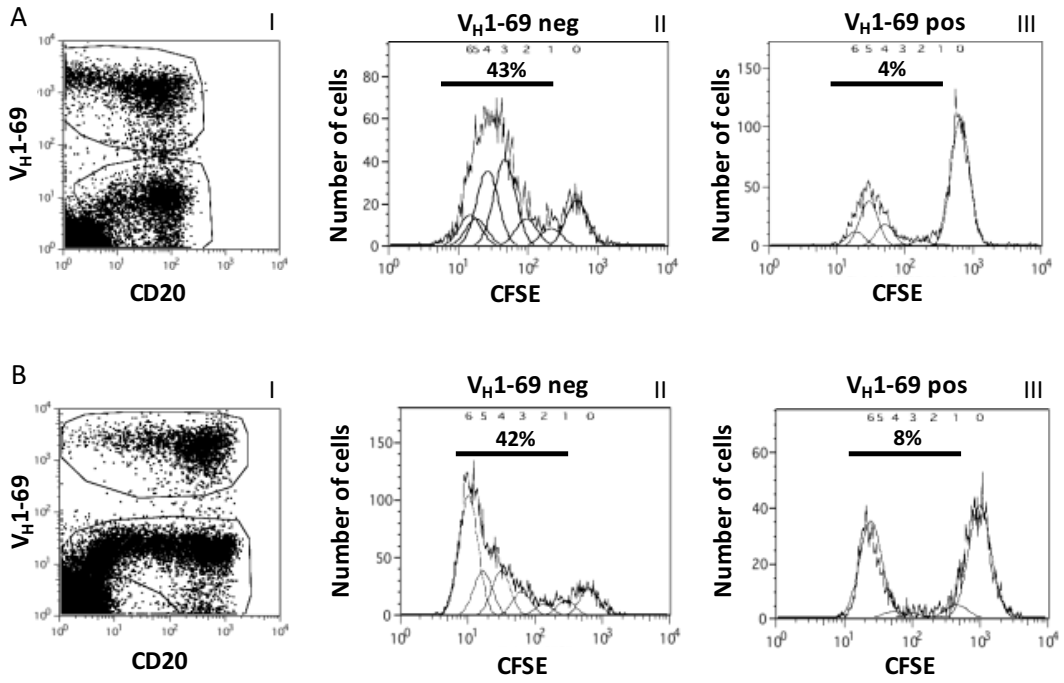


Figure 12. The V_H1-69$^+$ clonal B cells of HCV$^+$ MC patients fail to proliferate in response to TLR9 stimulation

Strategy for the analysis of the proliferative responses of V_H1-69$^+$ and V_H1-69$^-$ B cells before therapy (A) and at SVR24 (B) is shown. At the end of a 5-day culture in the presence of stimuli (CpG), CFSE labeled peripheral blood mononuclear cells (PBMCs) were permeabilized and stained with antibodies to CD20, IgM and V_H1-69 (or with mouse IgG as control). V_H1-69$^+$ and V_H1-69$^-$ B cells were analyzed for CFSE fluorescence (histograms). Percentages in the histograms denote the percent of divided cells (number of precursor B cells that have undergone at least one division).

This observation was confirmed in three other patients studied before antiviral therapy and at SVR24. The table 1 summarizes data concerning the proliferative responses of V_H1-69$^+$ and V_H1-69$^-$ B cells following stimulation with CpG (patient number corresponds to the Table 1 reported in *Materials and Methods;* findings in patient n. 7 are also reported in the figures 12 A-B).

Patient n.	V_H1-69-positive		V_H1-69-negative	
	Pre-therapy	SVR24	Pre-therapy	SVR24
3	1.7	3.7	33	26
7	4	8	43	42
15	2.7	4.1	31	30
22	1.26	3.5	34	38
Mean ± SD	2.4 ± 1.2	4.8 ± 2.1	35.2 ± 5.3	34 ± 7.3
Healthy donors 37.9 ± 7.7				

Table 1. Proliferative responses to CpG stimulation

The table summarizes data on the proliferative responses of V_H1-69$^+$ and V_H1-69$^-$ B cells to stimulation with CpG in 4 patients (n. 3,7,15 and 22 in the table 1 in Materials and Methods) studied before antiviral therapy and at SVR24; findings in one of these patients (n.7) are also reported in the figure 12 of this paragraph. Results in 12 healthy donors, in which whole CD20$^+$ B cells were gated for the analysis of proliferation, are also reported. Numbers in the Table denore the percentages of cells that started dividing (precursor cohort) after activation with CpG.

The number of V_H1-69$^+$ cells entering division (precursor cohort) in the 4 patients analyzed was 2.4 ± 1.2 in the pre-therapy and 4.8 ± 2.1 at SVR24, whereas it was 35.2 ± 5.3 and 34 ± 7.3, respectively, in autologous V_H1-69$^-$ cells. These findings indicate that, despite the reversion of the CD21^{low} phenotype, as indicated by the decrease in the proportions of V_H1-69$^+$ CD21^{low} cells from 84.8±9.9% in the pre-therapy to 41.5±12% at SVR24 (p=0.043), long-lived clonal B cells failed to restore their capacity to proliferate in response to TLR9 stimulation.

Chapter 5

DISCUSSION

Mixed cryoglobulinemia (MC) is the most frequent and well known extrahepatic manifestation caused by chronic HCV infection and characterized by systemic vasculitis and lymphoproliferation. In this disorder HCV induces the non-malignant monoclonal expansion of B cells producing a polyreactive natural antibody which is commonly encoded by the V_H1-69 and V_K3-20 genes and has both rheumatoid factor and anti-HCV activity [33,85]. The technical ability to identify isolate HCV-specific B cells through an anti-idiotype V_H1-69-specific antibody (G6 antibody) has made this condition an important model for studying humoral immune dysfunction during human chronic viral infection.

Our group and others [85] have previously shown that clonal B cells of patients with HCV^+MC display peculiar phenotypic and functional features: in particular, they commonly express low levels of CD21 ($CD21^{low}$ B cells), express a typical array of inhibitory and apoptosis-related genes and a distinctive pattern of homing receptors including CD11c, fail to flux calcium upon B cell receptor (BCR) triggering and to proliferate in response to the stimulation of BCR or of Toll-like receptor 9 (TLR9), and are highly prone to die by apoptosis [80,86,87]. Identical $CD21^{low}$ B cells are also expanded in patients with common variable immunodeficiency (CVID) [160] and in HIV-infected individuals [106]. These $CD21^{low}$ B cells have been defined "exhausted", rather than anergic, for their similarity with virus-specific exhausted T cells [106]; moreover, for some of their chatacteristics, human $CD21^{low}$ B cells recall the murine "aged B cells" (ABCs) increased in aged mice [118,119], which are $CD21^{low}CD11c^+$ B cells expressing the T-box expressed in T cells (T-bet) transcriptional factor and are important for the control of viral infections [120].

Just over 10 years ago, Gauld and co-workers [129], using a transgenic mouse model of B cell anergy, showed that constitutive ERK signaling and reduced lifespan are

reversed by the dissociation of self-antigen from the BCR, indicating the need for continual BCR occupancy for maintaining anergy. In 2014, Visentini et al. showed that $CD21^{low}$ B cells of $HCV^{+}MC$ and of CVID patients display high constitutive expression of the active phosphorylated form of the extracellular signal regulated kinase (pERK) [160]; this signature, together with reduced calcium flux and proneness to apoptosis, makes these cells closely resembling murine B cells made anergic by continual BCR engagement by antigen [135].

Usually MC regresses after eradication of HCV with interferon (IFN), whose immunomodulatory and anti-proliferative activities might contribute to this effect. In this study, I exploited the newly available Direct Acting Antivirals (DAAs), which rapidly suppress HCV viremia in $HCV^{+}MC$ patients and lack the immunomodulatory and anti-proliferative properties of interferon, to evaluate the impact of eliminating chronic antigen exposure on B cell behavior, untangling the effects of BCR disengagement in a human model of virus-driven anergy and exhaustion.

First of all, I evaluated and then confirmed that all the typical features of clonal B cells expanded in $HCV^{+}MC$, previously reported by my group [85], were also present in patients enrolled in this study. In particular, I observed that clonal B cells of most patients with untreated $HCV^{+}MC$ had high basal levels of pERK, while both BCR-induced ERK phosphorylation and the rate of increase in pERK after BCR ligation (fold increase) were reduced. Uncoupling of constitutive and BCR-induced ERK activation has been observed not only in $CD21^{low}$ B cells, confirming previous observation in $HCV^{+}MC$ [84] and CVID [160] patients, but also in their $CD21^{high}$ counterparts; furthermore, I noticed that $CD21^{high}$ B cells of $HCV^{+}MC$ patients showed a lower level of constitutive and BCR-induced ERK phosphorylation, but still higher compared to healthy donors. Patients' B also had increased apoptosis upon in vitro culture without stimuli and this proneness to die seems to be typical of $CD21^{low}$ B cells but not of their $CD21^{high}$ counterparts.

In addition to these anergy-like features [129,135], clonal B cells also displayed the features of exhausted B cells [80,86,87] as they were mostly $CD21^{low}IgM^{+}CD27^{+}CD11c^{+}FCRL4^{+}$ and failed to proliferate in response to BCR or TLR9 stimulation.

Four weeks after the beginning of DAA therapy, when all patients were negative for HCV RNA, constitutive ERK phosphorylation was significantly reduced compared to the pre-therapy levels, although BCR-induced ERK phosphorylation increased only slightly; this observation, obtained in patients' total B cells, was confirmed both in $CD21^{low}$ and $CD21^{high}$ subsets. At this time point, spontaneous in vitro apoptosis was also significantly reduced. The possibility that early normalization of constitutive ERK activation and apoptosis was due to the replacement of clonal B cells by normal B cells contrasts with the fact that the proportions of $CD21^{low}$ B cells in these patients were unchanged at week 4 of therapy.

These results support the idea that, as in the case of antigen-induced reversible B cell anergy in mice [129], reduction in the lifespan of B cells of HCV^{+}MC patients depended on chronic stimulation and could be reversed by disengagement of the BCR. Although it is believed that reduced lifespan of murine anergic B cells is largely due to the unfavorable competition for B cell-activating factor (BAFF) [126], the rapid increase of lifespan observed after BCR disengagement suggests that chronic BCR signaling is sufficient to initiate apoptosis [129]. Thus, I investigated whether reduced lifespan of MC B cells was related to ERK signaling; treating MC B cells with the MEK/ERK inhibitor U0126 failed to reduce spontaneous in vitro apoptosis, suggesting that ERK signaling is not directly involved in their pro-apoptotic pathway.

Monitoring clonal B cells phenotype over time, I observed that the proportions of $CD21^{low}$ B cells declined steadily up from baseline to SVR24, thus indicating a reversion of the $CD21^{low}$ phenotype, although they remained on average higher than in healthy donors. However, the proportions of V_H1-69-expressing B cells remained

stable up to SVR24 in 3 out of 6 patients, suggesting that phenotypic changes occurred in clonal B cells after eradication of HCV, as previously observed in HCV^{+}MC patients treated with IFN [164]. Despite phenotypic changes, I observed that long-lived clonal B cells failed to restore their capacity to proliferate in response to TLR9 stimulation. This indicated that HCV-driven B cell exhaustion makes use of durable reprogramming mechanisms irrespective of the CD21^{low} phenotype, as suggested by previous studies [89,161].

Recently, T-bet^{+} CD21^{low}CD11c^{+} B cells similar to murine ABCs were found increased in patients with chronic hepatitis C or cirrhosis without MC and, importantly, eradication of HCV with DAAs led to their decrease indicating a dependence on infection [121]. Although I didn't investigate and cannot exclude T-bet expression in clonal B cells of MC patients, their CD27^{+}IgD^{+}IgM^{+} B cells [33,85] are phenotypically distinct from the T-bet+ B cells of HIV-infected patients without MC, which are mostly CD27^{-}IgD^{-}IgM^{-}IgG^{+} class-switched cells [121]. Interestingly in this regard, while the T-bet^{+} CD27^{-}IgG^{+} B cells of HCV-infected patients decrease after antiviral therapy, the few T-bet^{+}IgD^{+}IgM^{+} B cells appear to increase [121]. No data to date specifically link the anergy properties observed in cryoglobulin-producing B cells and T-bet, but the phenotypic similarities mentioned above (CD21^{low}CD11c^{+}) suggest a possible relationship that should be explored; the rapid upregulation of T-bet observed in convalescent CD21^{low} B cells upon re-exposure to autologous HCV strains ex vivo [121], suggesting a link between BCR ligation, T-bet and the CD21^{low} exhaustion phenotype.

In summary, I show that the rapid clearance of HCV viremia with DAAs, which unlike interferon do not act as immunological modulators, offers a unique model for untangling the interplay of virus-driven anergy and exhaustion in human B lymphocytes. Clonal B cells of HCV^{+}MC patients display both signatures of anergy, induced by continual BCR occupancy, and of exhaustion driven by chronic viral infection; anergy features (pERK overexpression and accelerated apoptosis) rapidly

revert after disengagement from HCV, while phenotypic and functional features of exhaustion persist for several months.

Much of the results showed in this thesis have been recently published on *Blood* journal (*Del Padre et al*., July 2017) [162] and the paper was also featured in "This Week in Blood", a weekly snapshot of the hottest studies from each week's issue of *Blood*. In this comment, the author, David E. Kaplan (University of Pennsylvania), attributes a clinical relevance to these data, asserting that the persistence of $V_H1\text{-}69^+$ B cells after clearance of HCV viremia clearly explains the persistence of symptomatic cryoglobulinemic vasculitis observed in some HCV-cured patients [163].

In Kaplan opinion, the fact that B cell exhaustion phenotype is durably programmed into antigen-specific B cells suggests that B cell exhaustion might rapidly redevelop in the setting of reinfection, potentially contributing to the lack of protective immunity observed in many resolved chronic infections.

In the conclusion of the comment, the author claims that this study creates the context for further exploration of the mechanisms associated with induction of B cell exhaustion in vivo. Once the regulation of B cell anergy in chronic infection is better understood, therapeutic manipulation could significantly affect the outcomes not only of chronic hepatitis C, but also of other chronic viral and parasitic infections [164].

Chapter 6

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