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La infección persistente por virus de la hepatitis C (VHC) altera la reactividad de las células citotóxicas VHC específicas mediante el desequilibrio entre Mcl-1 y Bim debido a la disminución de la expresión de CD127.

TESIS DOCTORAL

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Abbreviations

2′-5′ OAS	2′-5′ oligoadenylate synthetase
ALT	Alanine amino-transferase
Apaf-1	Apoptosis protease-activating factor-1
Bcl-2	B cell lymphoma 2
BH	Bcl-2 homology
Bim	Bcl-2 interacting mediator
CHC	Chronic Hepatitis C
CTL	Cytotoxic T lymphocyte
DC	Dendritic cells
dsRNA	Double stranded RNA
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FOX03A	Fork-head box transcription factor
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C virus
HCVpp	HCV pseudo typed particles
IFN-γ	Interferon-γ
IL-7R	IL-7 receptor
IPS-1	IFN- β promoter stimulator protein 1
IQR	Interquartile range
IRES	Internal Ribosome Entry Side
IRF3	IFN regulatory factor 3
ISGs	IFN-stimulated genes
IU/L	International units per liter

KIR	Killer cell–Ig-like receptors
LCMV	Lymphocytic choriomeningitis virus
LSP	linfocitos de sangre periférica
LTC	linfocitos T citotóxicos
MAVS	Mitochondrial antiviral signaling protein
Mcl-1	Myeloid cell leukemia-1
mDC	Myeloid DCs
MFI	Mean fluoresence Intensity
Nabs	Neutralizing antibodies
ORF	Open reading Frame
PAMP	Pathogen Associated Molecular Patterns
PBL	Peripheral Blood Lymphocytes
PBMC	Peripheral blood mononuclear cells
pDC	Plasmocytoid Dendritic Cells
PDL-1	Programmed Death ligand-1
PE	phycoerythrin
Pe-Cy5	phycoerythrin Cyanine-5
PKC	protein kinase C
PKR	Protein kinase R
PP2A	Protein phosphatase 2A
pSTAT-5	STAT-5 phosphorylation
RIG-1	Retinoic acid–Inducible Gene I
RPMI	Roswell Park Memorial Institute
RT	room temperature
SR-B1	Scavenger receptor class B member I

SVR	Sustained virologic responder
TCR	T cell receptor
TGF-β	Transforming growth factor -β
TLR3	Toll like receptor-3
TRAF-1	TNFR-associated factor 1
Tregs	Regulatory T cells
UTR	Un-translated Region
VHC	Virus de la hepatitis C
VISA	Virus-induced signaling adapter

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I

RESUMEN /

ABSTRACT

Antecedentes: En la infección persistente por virus de la hepatitis C (VHC), la reactividad de los linfocitos T citotóxicos (LTC) se encuentra alterada y esto afecta al control del VHC. La expresión del receptor de la interleuquina-7 (CD127) en estas células podría regular la reactividad de los LTC a través de la modulación del equilibrio entre Bim/Mcl-1. Bim es una molécula pro-apotótica bloqueada por la acción de Mcl-1. La expresión de Bim/Mcl-1 y la reactividad de los LTC VHC-específicos se compararon en relación al fenotipo CD127.

Material y Métodos: Se obtuvieron linfocitos de sangre periférica (LSP) de pacientes HLA-A2^{+} VHC^{+}. Los LTC VHC-específicos se visualizaron mediante tinción de los LSP con Ac-anti CD8 y complejos pentaméricos HLA-A2/péptido (pentámeros). El fenotipo Mcl-1/Bim/CD127/IFN-γ en los LTC VHC-específicos se analizó mediante tinción de las células CD8^{+}/pentámero^{+} detectables con Ac anti Mcl-1/Bim/CD127/IFN-γ. La capacidad proliferativa in-vitro tras estimulación específica de los LTC VHC específicos se evalúo en presencia y ausencia del inhibidor z-VAD-fmk. Todas las células marcadas se analizaron mediante citometría de flujo.

Resultados: Los LTC VHC-específicos con fenotipo CD127^{bajo} se asociaron a elevada viremia del VHC, mientras que las células fenotipo CD127^{alto} se

correlacionaron con carga viral indetectable (P < 0.001). Directamente ex vivo, la frecuencia de células pentámero^{+} fue similar en los grupos con expresión alta y baja de CD127. Sin embargo, la capacidad prolierativa tras estimulación antígeno-específica se encontraba alterada en el grupo con expresión CD127^{bajo} (P<0.05), aunque esto se corrigió mediante tratamiento con z-VAD-fmk (P<0.05). La expresión de Mcl-1 directamente ex vivo fue baja (P<0.01) y la expresión de Bim aumentó tras el encuentro antigénico en las células con fenotipo CD127^{bajo} (P<0.05). La diferencia ex vivo entre la expresión de Mcl-1 y Bim en las células pentámero^{+} se correlacionó directamente con la expresión de CD127 (P<0.001) y con la reactividad de las células pentámero^{+} (P<0.05). La frecuencia de células productoras de IFN-γ directamente ex vivo fue menor en las células CD127^{bajo} que en las CD127^{alto} (P<0.05).

Conclusiones: Un expresión baja ex vivo de Mcl-1 y una sobre-expresión de Bim tras el encuentro antigénico se encuentran involucradas en la hiporeactividad de los LTC-VHC-específicos con baja expresión de CD127 durante la infección crónica, aunque esto puede ser superado mediante el bloqueo de la apoptosis.

Background: In persistent hepatitis C virus (HCV) infection, HCV-specific cytotoxic T lymphocyte (CTL) reactivity is impaired and this affects HCV control. Interleukin-7 receptor (CD127) expression on these cells could regulate CTL reactivity through Mcl-1/Bim balance modulation. Bim is a pro-apoptotic molecule blocked by the action of Mcl-1. Mcl-1/Bim expression and T cell reactivity on HCV-specific CTLs were compared according to CD127 phenotype.

Materials and Methods: Peripheral blood lymphocytes (PBL) from HLA-$A2^+$ HCV^+ patients were obtained. HCV-specific CTLs were visualized by staining PBL with anti-CD8 and HLA-A2/peptide pentameric complexes (pentamer). Mcl-1/Bim/CD127/IFN-γ phenotype of HCV-specific CTLs was tested by staining detectable $CD8^+$/$pentamer^+$ cells with anti-Mcl-1/Bim/CD127/IFN-γ antibodies. HCV-specific CTL proliferation ability after specific in vitro challenge was tested in the presence and absence of pancaspase inhibitor z-VAD-fmk. All stained cells were analyzed by flow cytometry.

Results: $CD127^{low}$-expressing HCV-specific CTLs associated with high HCV viraemia, while $CD127^{high}$ correlated with undetectable viral loads ($P < 0.001$). Directly ex vivo, $pentamer^+$ cell frequency was similar according to CD127 expression level. Nevertheless, $CD127^{low}$ $pentamer^+$ cell proliferation after

specific in vitro challenge was impaired ($P < 0.05$), although this was corrected by z-VAD-fmk treatment ($P < 0.05$). Mcl-1 expression was low directly ex vivo ($P < 0.01$), and Bim was up regulated after antigen encounter ($P < 0.05$) of $CD127^{low}$ $pentamer^{+}$ cells. The ex vivo difference between Mcl-1 and Bim expression on $pentamer^{+}$ cells correlated positively with CD127 expression level ($P < 0.001$) and with $pentamer^{+}$ cell reactivity ($P < 0.05$). The frequency directly ex vivo of IFN-γ-producing $pentamer^{+}$ cells was lower in $CD127^{low}$ group than in $CD127^{high}$ group ($P<0.05$).

Conclusion: A low ex vivo Mcl-1 expression and Bim up-regulation after antigen encounter are involved in $CD127^{low}$ HCV-specific CTL hyporeactivity during chronic infection, but it can be overcome by apoptosis blockade.

II

INTRODUCTION

Hepatitis C virus (HCV) is hepatotropic, non-cytopathic virus able to evade immune system efficiently as mechanism to persist in infected hosts. It has been estimated that more than 170 million people are infected with HCV. This virus is spread by contact with infected blood and body fluids. Approximately 80% of infections succeed in establishing a chronic infection with the potential for developing severe liver diseases such as cirrhosis and hepatocellular carcinoma (HCC) [1, 2].

No effective vaccine against HCV is available till date. Therapy for HCV infection as peg-interferon-alpha and ribavirin [3], has limited efficacy, in particular against the genotype 1 virus [4, 5]. The standard care for HCV genotype 1 is currently protease inhibitor (Boceprevir or Telaprevir), peg-Interferon-alpha and ribavirin while for other genotypes double therapy with peg-interferon alpha plus ribavirin is still the standard of care. An extended search for new therapy is progressing, already in phase III studies ready to come in the market in the next two years, but still many chronic HCV patients are not cured with these combinations. For these reasons it is necessary to look for new strategies to control HCV infection. Due to the lack of adequate cell culture systems, HCV studies have been slowed down for a long time, but continuous progress in the last few years it has overcome this obstacle. In-vivo model to study the biology of HCV have been significantly restricted due to the limited experimental availability of chimpanzees, the primary model for HCV [6, 7], and difficulties encountered in reproducing true infection in small animals. Two breakthroughs has been an important contribution to the field:

firstly, subgenomic replicons (i.e. without structural genes) [8-10], which are highly permissive for HCV replication [11] and secondly, HCV complete replication in cell culture [12-14]. However, it has long been recognized that these models are complicated by the particularly high error rate of the HCV RNA replicase [15].

Moreover, it is widely accepted that immune-mediated host-virus interactions are responsible for the outcome of HCV and pathogenesis of further severe diseases. Hence, it is interesting to deep in the knowledge of these interactions to find new potential theraputical targets. To be familiar with HCV infection, a brief outline of HCV structure and its life cycle is provided below.

II.1 HCV structure:

The hepatitis C virus (HCV) is an enveloped; positive stranded RNA virus and represents the Hepacivirus genus in the Flaviviridae family. HCV is a small, enveloped, positive sense single-stranded RNA-virus. Spherically shaped with a diameter of between 55–65 nm HCV virion consists of the HCV RNA genome, core and the envelope glycoproteins, E1 and E2. The HCV genetic material (RNA) surrounds by an icosahedral capsid of core proteins, which encased in a lipid membrane, called the viral envelope, in which are anchored the envelope glycoproteins E1 and E2, derived from host cell (Fig II.1) [16].

HCV Genome:

The positive sense single stranded RNA genome contains two highly conserved non-coding regions (UTR, un-translated region) in the region 5' and 3' which flank a coding region (ORF, open reading frame) that is 9600 base pair long [17]. At the 5' and 3' ends of the RNA are important to translation and replication of the viral RNA instead of translating into proteins. The 5' UTR site that is ribosome-binding site called IRES —Internal ribosome entry site, starts the translation of a very long protein containing about 3,000 amino acids [18]. Structural proteins of the HCV include Core protein, E1 and E2; whereas, NS2, NS3, NS4, NS4A, NS4B, NS5, NS5A, and NS5B as non-structural proteins are located at 3’ end and function as protease, helicase and polymerase activities necessary for viral replication [19] (Fig. II.1).

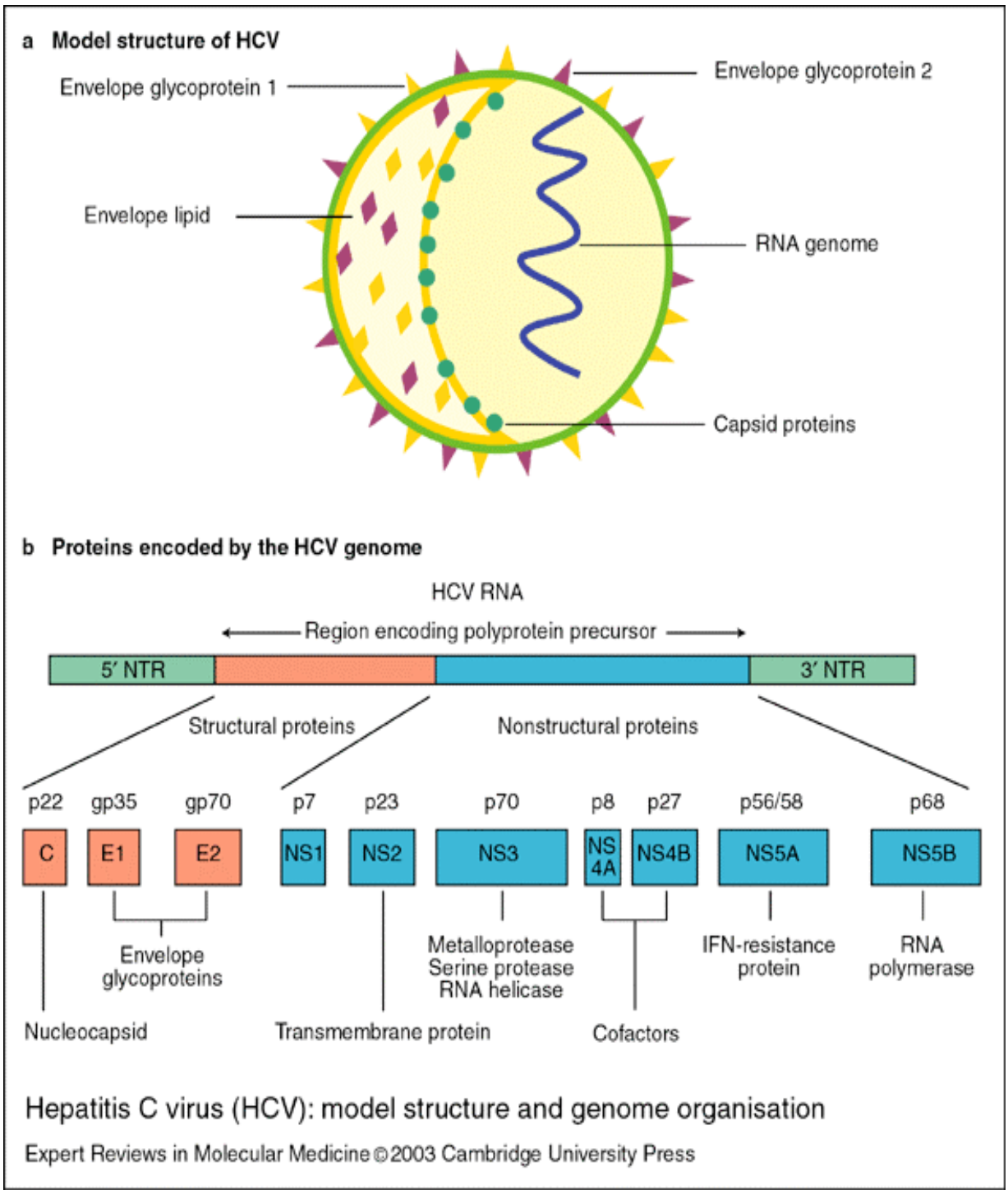


Figure II.1: Hepatitis C virus: model structure and genome organization.

Table II.1: Functions of genetic elements of HCV.

Genetic Element	Function
Regulatory sequences	
5′UTR	Internal ribosomal entry site for translation; replication
3′XR	Translation and replication
Viral proteins	
Core	Nucleocapsid; assembly
E1 and E2	Envelope proteins; assembly and entry
p7	Assembly†
NS2	NS2-3 protease
NS3	Serine protease, nucleotide triphosphatase, and RNA helicase
NS4A	Cofactor for NS3 protease activity; replication†
NS4B	Replication†
NS5A	Phosphoprotein; replication† and interferon sensitivity sequence‡
NS5B	RNA-dependent RNA polymerase

* 3′XR = 3′ untranslated region; 5′UTR = 5′ untranslated region.
† A proposed function.
‡ This idea is controversial.

T. Jake Liang etal Ann Intern Med. 2000.

HCV is very heterogeneous in their genome. This is due to the rapid viral replication, ranging between 10^{10} to 10^{12} virions per day (and a predicated viral half-life of 2 to 3 hours) [20] and high error rate of viral RNA polymerase (10-5 errors / nucleotide) without proofreading activity of RNA polymerase [21]. These all reasons are responsible to generate different genotypes, refers to the heterogeneity among different HCV strains isolated in different geographical areas and reflects the accumulation of mutations over a long period of evolution of the virus, and quasispecies, which are the translation of the heterogeneity arising during viral replication in the infected

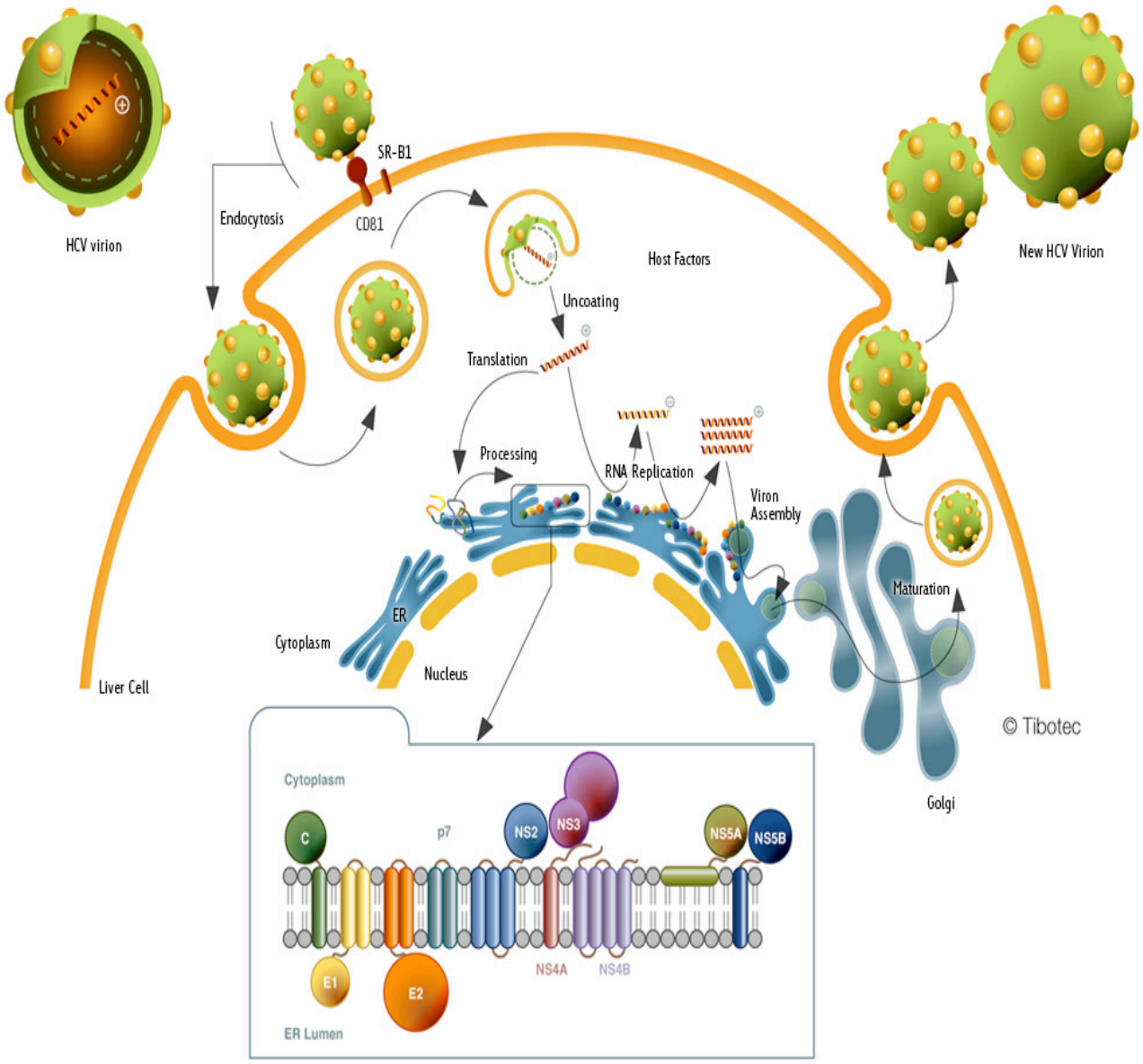
person [22, 23]. There are six known genotypes (numbered 1 through 6) and more than 50 subtypes (e.g., 1a, 1b, 2a etc) [24].

II.2 Life cycle of HCV

The full replication cycle of HCV can be done by using isolation and engineering of infectious HCV, which have been relatively recent realization in the field, as has the description and characterization of animal models for infection. The development of HCV replicons [8-10, 25], HCV pseudo typed particles (HCVpp) [26] and most recently the infectious HCV cell culture system [12-14] have advanced our understanding of the viral life cycle. Hepatocytes are the primary site of HCV infections. HCV life cycle begins with binding of the virus to cell surface receptors. The putative receptors, the tetraspanin protein CD81 [26-29], the scavenger receptor class B member I (SR-B1) [26, 30-32] and the tight junction proteins claudin-1 [33] and occluding, [34-36] have all been shown to enable HCV entry. In addition, the low-density lipoprotein receptor [28, 37-39], asialoglycoprotein receptor [40], and glycosaminoglycans (heparin sulfate) are also involved, but their exact roles have not been determined. By clathrinmediated endocytosis [41, 42], HCV enters the cell. The virus undergoes an uncoating process by fusion between the viral envelope and endosomal membrane in the acidified endosomal compartment [9, 29, 43-46] via E1/E2-mediated class II fusion [47, 48], to expose the viral genomic RNA to host-cell machinery. About ~9.6 kb viral RNA genome is released into the host cell cytoplasm, to serve as template for the translation of the viral proteins. IRES-mediated translation of

the HCV genome produces a single ~3,000 amino-acid polyprotein [49], which is processed by cellular and viral proteases into at least 10 different protein products. These products include the structural proteins, which form the viral particle (the virus core and the envelope proteins E1 and E2), and the nonstructural proteins P7, NS3, NS4A, NS4B, NS5A and NS5B [50]. Viral replication is driven by minus strand intermediate. HCV double stranded RNA (dsRNA) is freely exposed in the cytoplasm of infected cell [49], which is recognizable for host innate immune system.

Nucleocapsid is formed by assembling capsid proteins, genomic RNA. The nucleocapsid bud through intracellular membranes into cytoplasmic vesicles. Finally, by secretory pathway, mature enveloped virions release from the cell (Fig.II.2).



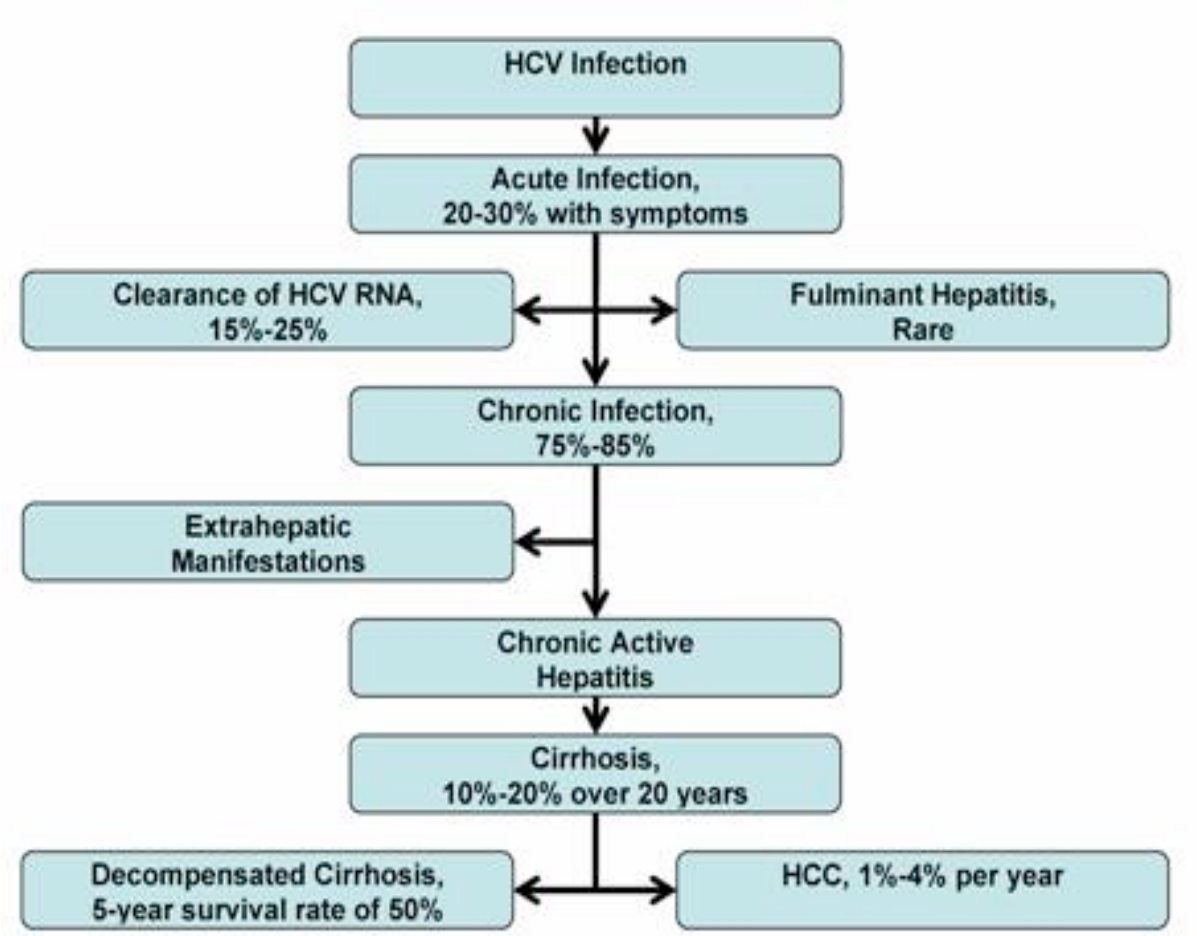
Tibotec, Belgium

Figure II.2: HCV life cycle

II.3 Natural history of HCV:

Acute HCV infection is difficult to diagnose because of asymptomatic behavior of 70%-80% of infected individuals. Most infected persons do not get diagnosed until many years later, due to unaware of their exposure to HCV. Chronic hepatitis C infection is marked by the persistence of HCV RNA in the blood for at least 6 months after onset of acute infection. Self-limiting infection in 15%-25% of patients have been observed. Approximately 75%-85% of infected patients fail to clear the virus by 6 months, and develop chronic hepatitis. Many factors affect the rate of chronic HCV infection, including the age at time of infection, gender, ethnicity, and the development of jaundice during the acute infection.

Therefore, a large proportion of HCV-infected persons, are at risk for advanced liver fibrosis, HCV- related extrahepatic complications, cirrhosis and HCC (Fig II.3). Moreover, a highly variable rates of liver fibrosis progression is the result of the amount of alcohol consumption, age of initial HCV infection, degree of inflammation, HIV or HBV co-infection, and co-mordid conditions. An estimated 10%- 20% of chronic HCV infections develop end-stage liver disease over one or two decades. During chronic HCV infection or cirrhosis, extrahepatic manifestations can appear and HCC can occur only after establishment of cirrhosis.



Stephen etal, Int. J. Med. Sci. 2006

Figure II.3: Natural history of HCV infection

II.4 Immune response:

To fight against a viral infection the host displays two kinds of immune responses: the innate and adaptive responses. The innate response is the first immunological barrier and it is essential in cytopathic viruses. This response limits viral spreading but also acts as adaptive response activator through antigen presentation to viral specific cells. Adaptive response is the second line in the immunological defense. It plays a major role in non-

cytopathic viral infections because this type of viruses behaves as an intracellular parasite and they remain occult to the innate system.

But, highly productive and replicative viruses such as, HCV is associated with ineffective antiviral immunity during persistent viral infections. The complex ineffective immune response involves the functional deterioration of antiviral responses and contraction of the size of this response (Fig II.3).

II.4.1. Innate immune response during acute HCV infection

The first response to HCV protein is thought to be IFN-β production by infected hepatocytes, which are able to secrete type I IFN. The infected cells are sensed with pathogen associated molecular patterns (PAMP), Toll like receptor-3 (TLR3) [51] and retinoic acid–inducible gene I (RIG-I) [52, 53] by endosomal dsRNA and cytosolic dsRNA respectively, which is an essential intermediate in the HCV replication cycle, and thus, they may be important in the pathogenesis of hepatitis C [54]. RIG-I recruits IFN- β promoter stimulator protein 1 (IPS-1; also called CARD adaptor inducing IFN-β CARDIF), virus-induced signaling adapter (VISA), and mitochondrial antiviral signaling protein (MAVS) [55-57], after ATP-driven activity dependant on recognition of viral proteins [58]. On other hand, TLR3 dimerization, due to leucine-rich repeats [59], recruits the adapter protein, Toll–IL-1 receptor domain– containing adaptor inducing IFN-β (TRIF). Both processes result in downstream signaling, nuclear translocation of IFN regulatory factor 3 (IRF3) and leads to

stimulation of the transcription of a set of genes including IFN-β [60]. Antiviral state, induced by secreted IFN β, gives an alert to uninfected cells by activation of effector molecules. Binding of IFN -β to cognate receptor complex leads to the activation of JAK/STAT pathway, which results in the induction of IFN-stimulated genes (ISGs) and leads to enhance the IFN response [61] (Fig. II.4).

However, HCV has organized a number of countermeasures not only to inhibit the induction phase, but also interfere with the effector phase of the IFN system (Fig. II.4). It has been confirmed, by in-vitro studies, that HCV serine protease, NS3/4A is enable to cleave MAVS [62], TRIF [63], IPS-1 [64] and oligomerization of MAVS, which is part of signaling process [51, 55, 62, 63, 65, 66]. Disruption of IRF-3 activation occurred by NS3 protein action [67] and it has been shown with different cell lines in-vitro studies [51, 66]. Another key player, HCV core, when over expressed in cell culture, disturbs antiviral activity via interfering in JAK/STAT signaling and ISG expression by inhibition of STAT1 activation. Simultaneously it induces its degradation [68, 69] by induction of inhibitor of the JAK/STAT pathway SOCS3 [70], protein phosphatase 2A (PP2A), which ultimately reduces the transcriptional activity of ISG factor 3 (ISGF3) [71]; and inhibition of ISGF3 interaction to IFN-stimulated response elements [61]. HCV NS5A interferes with the function of ISGs by inhibiting 2′-5′ oligoadenylate synthetase (2′-5′ OAS) and leads to overall ISG expression impairment [72]. Protein kinase R (PKR) can negatively regulate HCV replication noncytolytically in cell cultures [73, 74], which can interacts with HCV NS5A and lost its function. Interestingly, HCV

E2 acts as distraction target to PKR [75]. To sum up, the main targets of HCV proteins to evade immune response are interference with the induction of IFN synthesis, IFN-induced intracellular signaling and IFN-induced effector mechanisms (Fig.II.4).

Dendritic cells (DC) are professional antigen presenting cells with important functions in antiviral immunity through activation of adaptive immune responses. Type-I IFNs are also produced by plasmocytoid (p) DCs, which derive from the lymphoid lineage. Although, production of IFN alpha/beta, in early phase of infection occurs after recognition of ssRNA and dsRNA by TLR7 and TLR9 respectively, the mechanism is still not clear [76]. The frequency of pDCs in the blood [77] and their production of IFN-α in HCV infection are reduced after in vitro stimulation [78]. The possible mechanism has been demonstrated in in-vitro studies. First, HCV core and NS3 activate monocytes by TLR2 signaling to produce TNF-α [79], which in turn inhibits IFN-α production and induces pDC apoptosis [78]. Second, HCV itself inhibits IFN-α production of pDCs [80]. However, other studies revealed regular response to TLR stimulation by circulating pDCs of chronically infected individuals [81, 82] and they have high levels of endogenous type I IFNs without immuno-dysfuction [76]. Although this defense mechanism is significant, the host rarely overcomes HCV infection, which suggests several other viral evasion mechanisms that are poorly or not understood yet.

Another group of DCs, myeloid DCs (mDCs) derive from the myeloid lineage [83, 84]. Due to its tolerogenic and stimulatory role [83, 84],

mDCs have been broadly studied in HCV infection. mDCs have not been observed to be decreased in peripheral blood or dysfunctional in HCV chronic infected individuals in in-vitro studies [85, 86]. Nevertheless, HCV proteins can interact with monocytes/macrophages through TLR2, inducing the IL-10 production, which hampers IL-12 production by mDC and IFN-α by pDC, or they directly inhibit DC differentiation [87]. IL-12 cytokine production by mDC is decreased in HCV patients in response to stimuli like CD40 L or poly (I:C) [88], which can explain clearly the shift from Th1 to Th2 response in HCV patients. *In-vitro* studies indicate that DC expressing core and E1 proteins have lower stimulatory ability, which is associated to the lack of maturation after stimulation with TNF-alpha or CD40L [89].

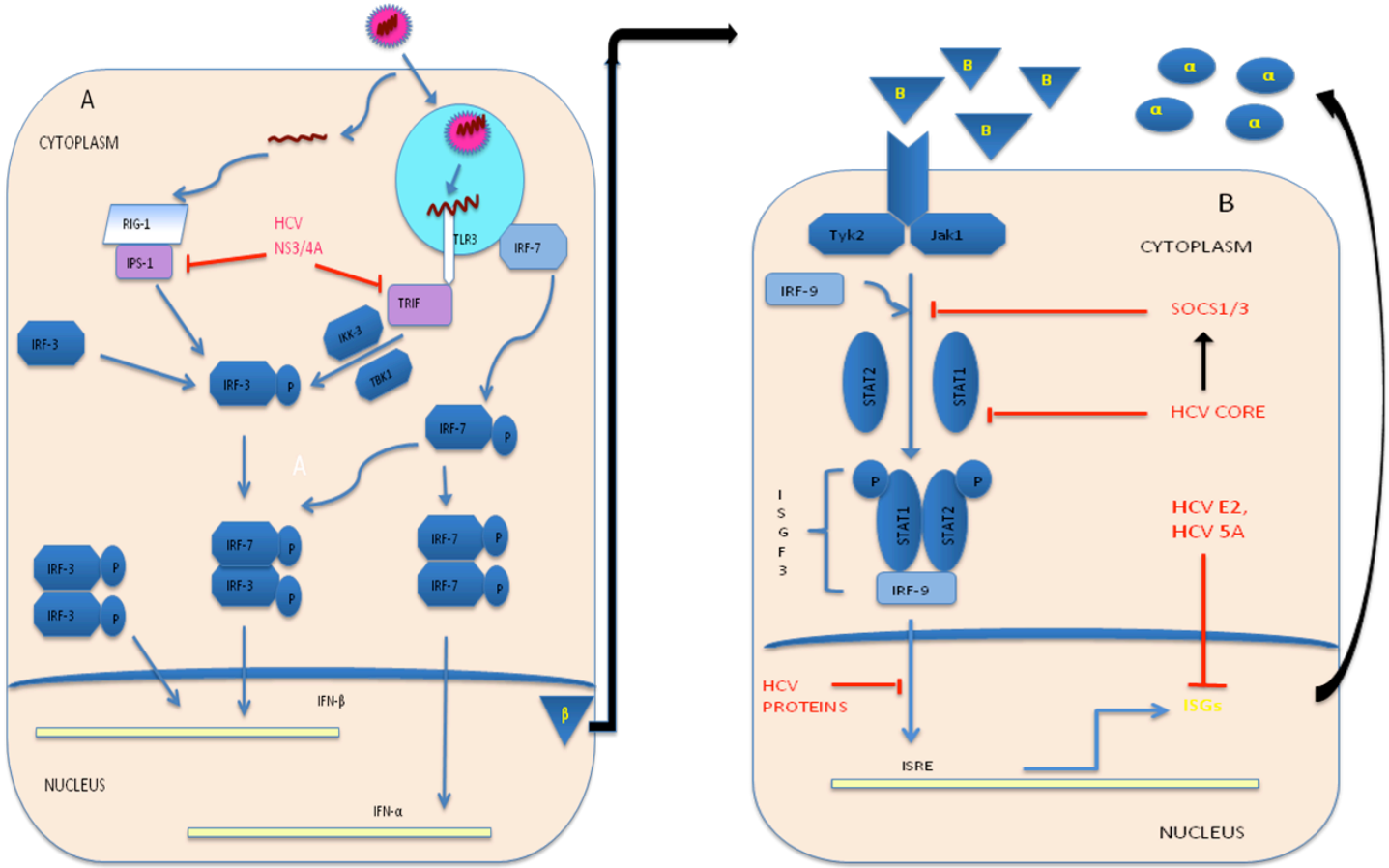


Figure II.4: Evasion of Innate immune response by HCV: (A) Interference in IFN synthesis: Blocking of TLR 3 and RIG-1 signaling respectively, by cleavage of the adaptor molecule TRIF and IPS-1 via HCV NS3/4A; (B) Interference in IFN-induced Effector mechanisms: Binding of IFN β and its receptor with TYK2 and JAK1 kinase activation lead to form ISGF3 complex, where this complex interact with IFN stimulated response elements (ISREs) within the promoter and enhancer region of ISGs to induces ISGs (such as 2', 5' OAS, PKR, IRF7) production in nucleus. HCV core induce SOCS1/3, which is the inhibitor of the JAK/STAT pathway and inhibits STAT1 phosphorylation, which inhibits assembly of trimeric ISGFs complex. Function of ISGs inhibited by HCVE2 and HCV NS3/4A.

Other cells involved in the innate response are the NK cells. Functions of these cells include generating a cytotoxic response, regulatory cytokines production and control on DC maturation and amplitude of DC response, which may deeply impact on type of down- stream adaptive immune responses. Response to HCV infection by NK cell is direct apoptosis induction of infected cells with production of antiviral cytokines [90, 91]. Moreover, NK cell depletion or dysfunction favor HCV persistence [90]. The role of interactions between HLA class I and killer cell–Ig-like receptors (KIR) during HCV infection has been shown. KIR can regulate NK cell activities. However puzzling contradictions for this topic in different studies have been revealed [92-94]. The importance of NK cells in the resolution of HCV infection is illustrated by the influence of genetic polymorphisms of KIR and their HLA ligands on the outcome of HCV infection, which was dependent on a homozygous HLA class I ligand background [95-97]. There is need to focus on clear understanding of functional and molecular HLA-KIR interactions to know about the possible way for NK cell- mediated protection in animal models of HCV infection.

However, an increased proportion of NK cells expressing activating receptors, enhanced cytotoxicity and defective cytokine production have revealed in chronic HCV infection [98]. Megan et al revealed that IL28A cytokine could significantly inhibit IFN-γ production lead to NK cell inactivation [99], which would be important to attenuate chronically activated NK cells. Consequently, the analysis of functional scene between NK cells and type 3 IFN in the immune response to virus will be required to understand the role of the NK in disease progression during HCV infection.

II.4.2. Adaptive immune response

The second barrier to control HCV infection is the adaptive immunity. This response has two arms to fight against pathogens; humoral and cellular immune response. Humoral immune response, that means neutralizing and non-neutralizing antibodies can endorse antiviral activity and pathogenesis [50]. Cellular immune response shows antiviral immunity by means of virus specific CD8 cytotoxic T lymphocytes (CTLs) and CD4 T helper cells, which play key effector and regulatory roles respectively. These T cells take part in viral pathogenesis of HCV by direct killing of infected cells or producing soluble factors able to clear the virus in a non-cytolytic manner, but also can lead to HCV pathogenic events, favoring direct liver damage and attracting non-specific inflammatory cells to perpetuate the liver inflammation [50].

II.4.3. Humoral immune response

Neutralizing antibodies (nAbs) generally play a critical role for controlling initial viremia and protecting from re-infection in viral infections. However, the role of the humoral immune response in the clearance of HCV infection has been in the dark for a long time due to difficulties to determine relative role of antibodies to neutralize HCV. It can exclusively be evaluated by relevant model systems. It is thought that HCV clearance could occur in the absence of nAbs. If they are present alone, these antibodies are inadequate to eradicate HCV in most of the cases in early studies [100-104]. It has been proved that HCV specific T cells may compensate for lack of neutralizing antibodies to obtain HCV clearance [105]. However, due to the development

of novel model systems [12-14, 26, 106], it is possible to focus on HCV entry into host cells and neutralization process which demonstrated that nAbs are induced by patients who subsequently control [107] or resolve [108] viral infection in the early phase of infection and contrary in chronic infection. This suggests that a strong, early, broad nAbs response may contribute to resolution of HCV in the acute phase of infection while delayed induction of nAbs may contribute to development of chronic HCV infection.

Instead of the rapid, vigorous and multi-specific antiviral host immune responses, chronic patients have been shown to develop a delayed and inefficient neutralizing antibody response [108] due to HCV escape mechanism [109]. Recent studies make evident that entry of HCV can be hampered or modulated by nAbs of chronic HCV patients [110-112], while it is controversial in cell culture study [113]. In addition, although nAbs are incapable to clear the virus in chronic infection, due to selection pressure exerting on viral variants, they contribute to the evolution of the HCV envelope sequences to escape [114, 115]. It has been proposed that HCV stimulates B cells in a B cell receptor-independent manner in chronic infection [116] and may favor the development of lymphoproliferative and autoimmune diseases [50]. Although, in vitro studies make evident that the neutralization ability of HCV-specific nAbs is enhanced by complement activation against pseudotyped viruses [116], there is absence of direct experimental evidence about the presence of any of these Ab- mediated functions during natural HCV infection. However, immune complexes are believed to play a pathogenetic role in the development of manifestations such as

cryoglobulinemia, glomerulonephritis, porphyria cutanea tarda, and necrotizing cutaneous vasculitis during chronic HCV infection [117-119].

II.4.4. Cellular immune response

Cytotoxic T lymphocyte (CTL) responses are essential to control HCV infection. Efficiency of antiviral CTL responses depends on where these cells are primed. Efficient antiviral CTL response is observed when it is primed in lymphoid organs, whereas within the liver, priming is more tend to induce T cell inactivation, tolerance or apoptosis [50]. A strong, multispecific and long-lasting T-cell immune response emerge to be important for control of viral infection [104, 120]. Persistent HCV unsuccessfully control by T effector cells is due to multiple causes, such as: HCV escape mutant generation, immunosuppressive effects exertion, Tregs induction, or effector T cell exhaustion or apoptosis [121-124].

II.4.5 Adaptive cellular response during acute HCV infection

Vigorous $CD4^+$ and $CD8^+$ T cell responses targeting multiple HCV regions with intrahepatic production of IFN-γ emerged in acute hepatitis C infection [101, 123, 125]. Decreasing viral titer correlates precisely with the appearance of HCV-specific T cells and IFN-γ expression in the liver [126]. The appearance of HCV-specific T cells can be detectable in the peripheral blood or in the liver compartment several weeks after infection in humans or experimental chimpanzee models [61, 104], respective with primary peak of

transaminases and irrespective of clinical outcome (resolution or chronicity). Delayed emerging of antigen-specific responses are also essential for the HCV control [61].

The protective function of $CD4^+$ T cells appear to be due to the production of antiviral cytokines, but also their helping nature to antiviral B cells and in $CD8^+$ T cell response. The HCV clearance has been observed and correlated with vigorous proliferation of specific $CD4^+$ T cells [80, 127] with concurrent IL-2 and IFN-γ production [128, 129]. The early sustained development of $CD4^+$ T cell response needs to be successful for viral clearance [128], whereas HCV- specific $CD4^+$ T cell responses are not observed in chronic HCV infection. Moreover, the recurrent viremia has been correlated with loss of previous strong $CD4^+$ T cell responses after several months of viral clearance [130, 131]. Studies on the relative importance of CD4 help in spontaneous recovery in acute HCV infection demonstrated that fact [132]. CTL priming in presence of CD4 help is critical factor in protective function [128].

Similarly, Antigen-dependent reactivity of HCV-specific $CD8^+$ T cells has been proved by a rapid decay of $CD8^+$ T cell responses during antiviral therapy [133]. It is evident that, CD8 T cells play a direct role in terminating hepatitis C as shown by prospective studies of acute infection in chimpanzees [134], in which it is revealed that a vigorous, multispecific, and polyclonal intrahepatic CD8 CTL response during early infection correlated with HCV-1 clearance. Furthermore, it is concluded after monitoring the course of HBV

infection in CD8-depleted in chimpanzees that $CD8^+$ cells are the main effector cells responsible for viral clearance and disease pathogenesis during acute HBV infection, and this study suggests that viral clearance is mediated by both noncytolytic and cytolytic effector functions of the $CD8^+$-T-cell response [135]. In addition, after accidental needlestick exposure, subjects who develop acute HCV infection, the vigor and quality of the antiviral T cell response determines the outcome of acute HCV infection. The subjects who cleared the virus experience a prolonged episode of acute hepatitis that coincided with a $CD8^+$ T cell response to HCV, while chronic infection develops in subjects who fail to produce a significant T cell response [123]. Therefore, significant, broader, multi-specific HCV specific CTLs are important in acute HCV infection control.

The appearance of self-sustaining memory T cells ($CD127^+$ memory HCV-specific $CD8^+$ T cells and $CD4^+$ T cells) are necessary to control HCV infection [101, 123, 128]. In fact, years after HCV control due to anti-HCV treatment it is possible to find HCV traces in association with HCV-specific T cell reactivity. These data suggest that HCV-specific memory T cells are essential to clear HCV infection completely after the initial acute clearing [136].

II.4.6 Adaptive cellular response during chronic HCV infection

Therefore, complete resolved HCV patients exhibit broader CTL responses with higher functional avidity and wider cross-recognition ability

than patients with persistent HCV infection [137]. There are evidences that demonstrate rapid mutation in HCV genome, T cell exhaustion because of expression of inhibitory molecules, immune regulatory cytokine induction and immune modulatory T reg cell activation, which are the main reasons for HCV persistence in chronically infected patients [121, 138-141]. Like Retrovirus, HCV polymerase has high replication rate and lack of proofreading capacity, which permit a rapid virus escape from emerging humoral and cellular immune responses and lead to persistent infection [139, 142]. Mutation study in early HCV infection in HLA class I restricted epitopes targeted by $CD8^+$ T cells are associated with persistence [143, 144], which proved indirectly that HLA-restricted $CD8^+$ T cells exert selection pressure. Furthermore, the HLA alleles can influence infection outcome [145].

The secretion of certain immuno-regulatory cytokines is also related with HCV persistence. IL-10 cytokine is found to increase during chronic HCV infection [146]. In chronic HCV patients, the suppression of IFN-γ production and proliferation of virus- specific $CD4^+$ and $CD8^+$ T cells have been observed in livers with IL-10 –producing HCV- specific $CD8^+$ T cells [147]. IL-10 produced by monocytes or NK cells down-regulates effector T cell responses. For instance, monocytes secrete IL-10 in response to HCV core–mediated TLR2 stimulation in vitro [148]. IL-10 producing HCV-specific $CD8^+$ T cells inhibits IFN-α production [149], but also promotes apoptosis of pDCs [148], and induces liver infiltration of chronically infected individuals, suggesting that they modulate liver immunopathology to favor HCV persistence [147]. In addition, intrahepatic HCV-specific IL-10 producing $CD8^+$ T cells prevent liver

damage during chronic disease [150]. Moreover, TGF-β is also involved in antiviral immune suppression and chronic HCV infection evolution [151]. To sum up these data, regulatory cytokines such as IL-10 or TGF-beta decrease liver inflammation, after affecting the protective immune response, developing a dual task. First of all, they impair T cell responses to allow viral persistence but also decrease liver damage to extend host survival.

Regulatory T cells (Tregs) are important to control the balance between host damage and viral control produced by specific immune response. In cases of excessive immune response, that could be harmful for the host, these cells can induce immune-tolerance to the viral epitopes. Tregs are derived from natural or induced T cell populations, in which natural $CD4^+$ Tregs are generated during normal T cell development in the thymus, whereas induced Tregs are generated from mature T cells [152]. T cell subset with suppressive function, $CD4^+$ $CD25^+$ $FoxP3^+$ regulatory T (Treg) cells, engages in the control of auto-immunity and immune responses, through various mechanisms including the inhibition of APC maturation and T-cell activation [153]. No difference has been found in the frequency of Treg cells and the extent of suppression irrespective of the outcome of the infection [154]. However, higher Tregs frequency has been observed in chronic HCV infected patients than in resolved patients [155-158]. Interestingly, depletion of $CD25^+$ cells could enhance responsiveness of the remaining HCV-specific effector cells in vitro [155-157], which suggests a fundamental role of Tregs in the establishment of chronic HCV infection. Moreover, Treg cells are induced and proliferate in chronic HCV infection and appeared to alter liver

inflammation [159]. Conversely, Programmed Death ligand-1 (PDL-1) mediated inhibition limits the expansion of Tregs by controlling STAT-5 phosphorylation (pSTAT-5) [160], which can diminish suppressive function of Tregs, lead to viral load control and ultimately ensure long-lasting survival of the host.

HCV is able to induce the up-regulation of different negative co-stimulatory molecules in order to provoke an anergic status on HCV-specific T cells. Expression of the inhibitory receptor PD-1 is one of these molecules involved in the generation of a state of exhaustion on HCV-specific $CD8^+$ T cells during chronic HCV infection [161, 162]. Importance of expression of PD-1 in HCV-specific T cell failure mechanism has been observed [163, 164], which can hinder by mutation in T cell epitopes [165]. In addition, blocking of PD-1 signaling resulted in the functional restoration of blood-derived HCV-specific $CD8^+$ T cell responses in chronic infection [164, 166]. However, the PD-1 alone is not sufficient in defining exhausted HCV-specific $CD8^+$ T cells during HCV infection. To restore function of HCV-specific T cells isolated from liver biopsies of infected patients, there is need of CTLA4 blockade in addition to PD-1 blockade [167]. In addition, the co-expression of other inhibitory receptors such as 2B4, CD160, Tim-3 and KLRG1 occurred in about half of HCV-specific $CD8^+$ T cell responses and correlate with low or intermediate level of CD127 expression, impaired proliferative capacity, and an intermediate T cell differentiation stage [168]. These data indicates that HCV infection modulates different negative co-stimulatory molecules to favor the development of HCV-specific $CD8^+$ T cell exhaustion.

T cell stimulation in the liver encourages tolerance by using mechanisms such as, immune divergence [169], generation of regulatory T cells [170], T cell anergy [171] and T cell death [172]. Undeniably, hepatic tolerance can explain the elevated frequency of viral persistence during hepatotropic virus infections [172]. Although there are evidences showing that most infectious microorganisms are promptly removed from the liver by inflammation, a favorable situation for evading immune responses occurs in some viruses, leading to the triumph of certain pathogens such as HBV and HCV. The Fig I.5 depicted inflammatory and tolerance activity in liver by stimulation of different molecules or receptors. Till date, there are two main mechanisms by which HBV and HCV could successfully escape from CTL action: escape mutant generation, and immunosuppressive effects exertion (effector T cell exhaustion and T cell death by apoptosis) [123, 124, 173, 174].

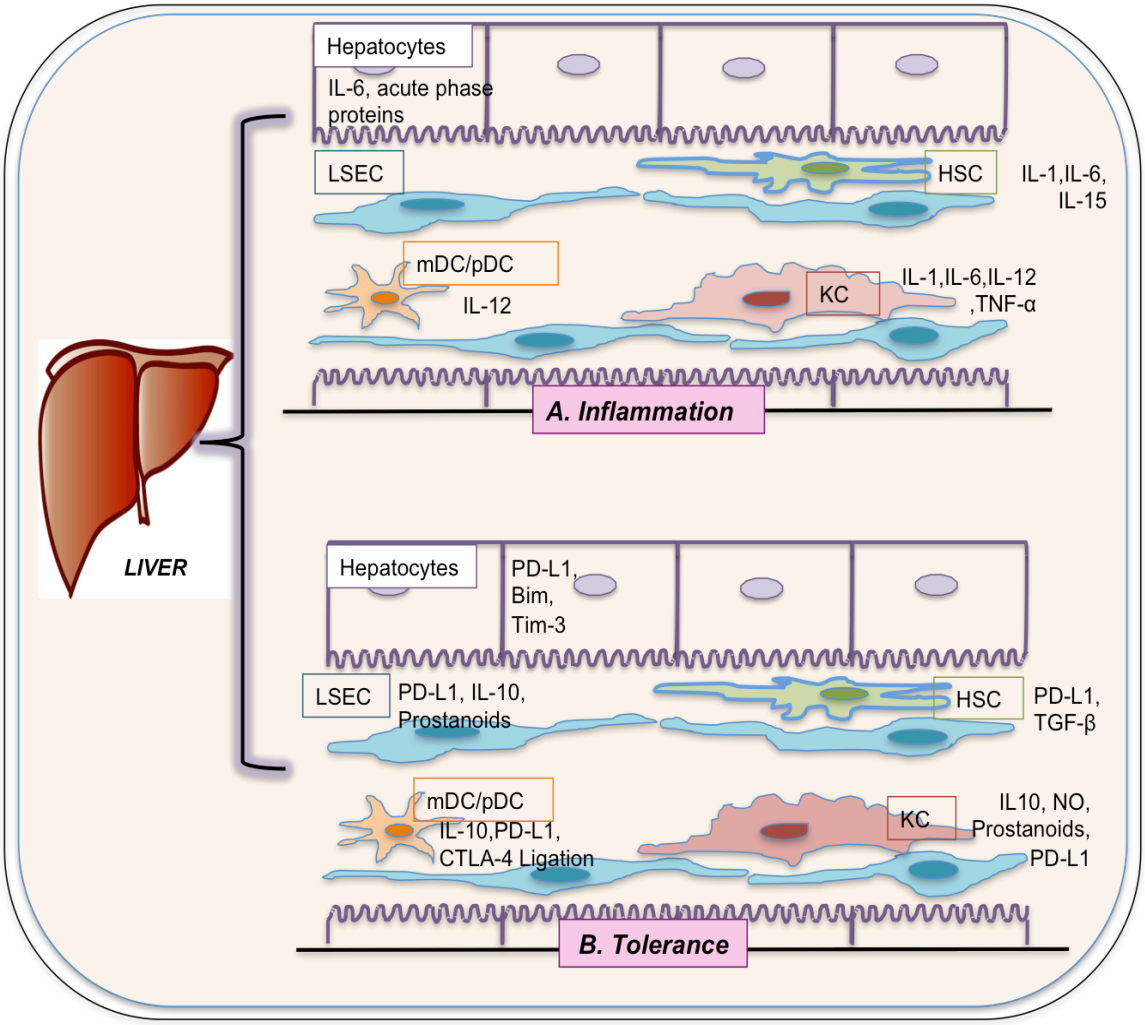


Figure II.5: Collective illustration of the hepatic cells with inflammatory and tolerance activities by stimulation of different molecules or receptors. LSEC: Liver sinusoidal endothelial cells; KC: Kuffer cells; DC: Dendritic cells; HSC: Hepatic stellate cells; TNF: Tumor necrosis factor; IL: Interleukin; mDC: Myeloid dendratic cell; pDC: Plasmacytoid dendritic cell; PD-L1: Programmed death ligand-1; Bim: BCL-2 interacting mediator; Tim-3: T cell immunoglobulin mucin-3; CTLA-4: Cytotoxic T-lymphocyte antigen 4; TGF: Transforming growth factor; NO: Nitric oxide.

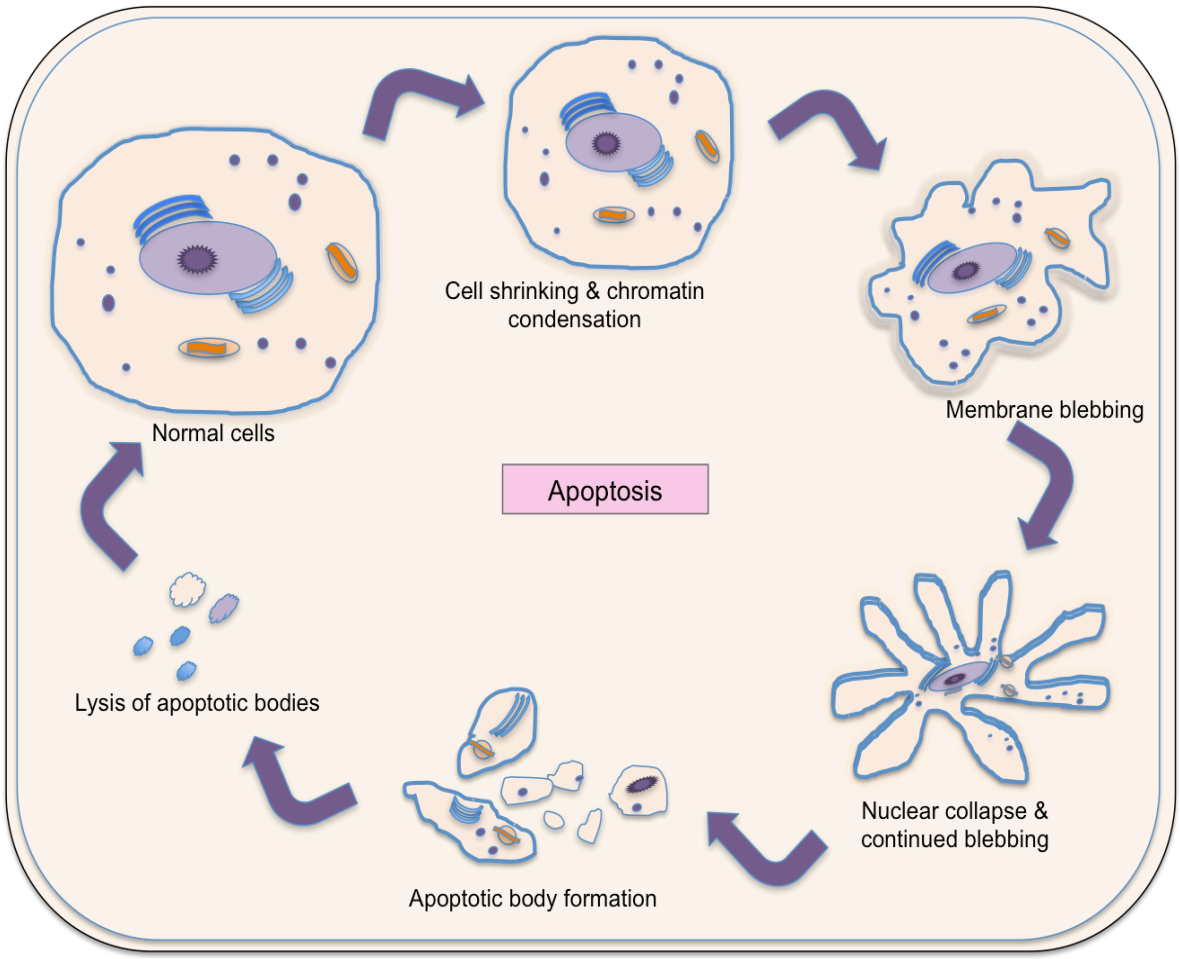


Fig II.6: Apoptosis-programmed cell death.

Among these mechanisms involved in viral hepatitis persistence, new advances on the role of T cell death induction have been obtained recently and will be discussed in the following pages.

II.5. Apoptosis:

A normal cellular process involving physiologically relevant cell death and deletion of unwanted cells is called apoptosis. Apoptosis is essential for cell selection, tissue homeostasis, morphogenesis, and host defense in multi-cellular organisms. A cell that undergoes apoptosis dies neatly, without

damaging its neighbors. The apoptotic signals give rise to activate various proteins and follow a specific classical caspase chain reaction set activation [175]. Quickly and neatly dismantlement process includes membrane blebbing with shrinking of the cytoplasm and condensation of the nucleus. Phagocytic cells begin to pick up the apoptotic bodies, preventing the release of cellular content and ultimately avoiding inflammation [176] (Fig II.6). Apoptosis occurs by two mechanisms: active and passive mechanism. No presence of antigen gives a signal for termination of immune response by passive apoptotic mechanism (intrinsic pathway). On the other hand, the ligation of Fas (CD95) and TNF receptors-“death receptors” triggered apoptosis lead to active mechanism of apoptosis (extrinsic pathway). Briefly, apoptosis mechanisms involve a family of cysteine proteases, called caspases. These molecules are synthesized in the cell as inactive precursors, or pro-caspases for self-protection against accidental death, which are usually activated after receiving proper trigger by cleavage (Fig II.7).

Structurally, pro-caspases contain three domains: N terminal prodomain, a large subunit and a small subunit. After activation, the active caspase enzyme is formed by heterodimerization of small and large subunits [177]. Moreover, active caspase molecules are ready to cleave target proteins such as structural or signaling proteins and other effector caspases, preventing other proteins cleavage randomly [176].

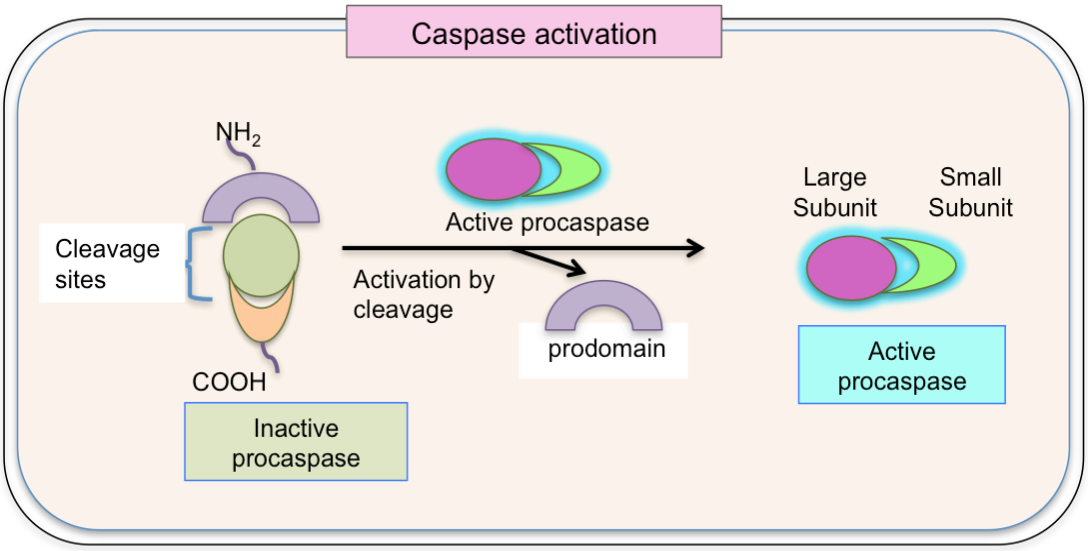


Figure II.7: Caspase activation: Inactive proenzyme (procaspase) activated by proteolytic cleavage by another member of caspase family and cleaved two fragments associate to form the active site of the caspase.

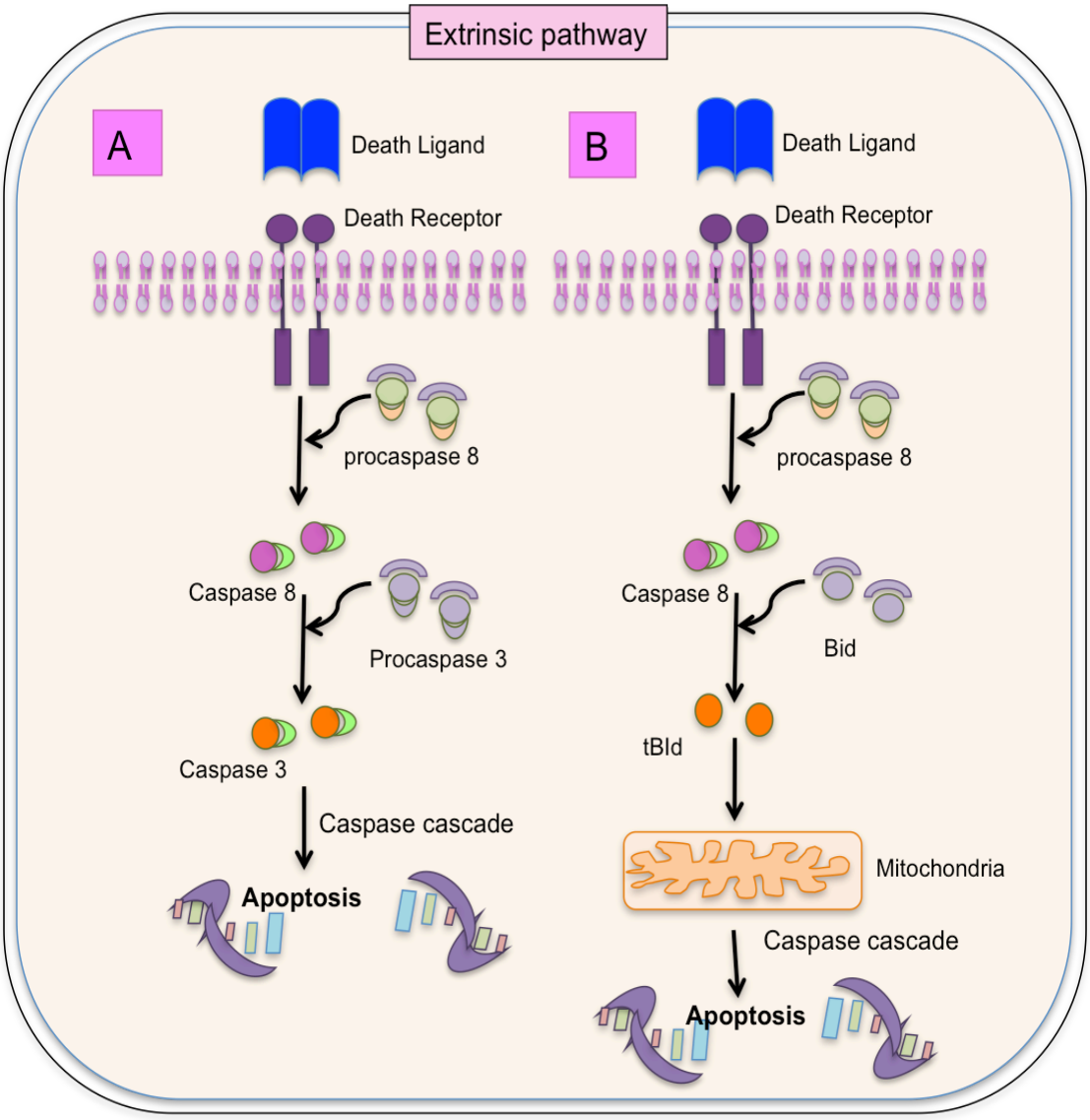


Figure II.8: Apoptosis: Extrinsic pathway. A: Mitochondria-independent extrinsic pathway: Fas-FasL ligation strikes to recruit pro-caspase 8 activation and induction of caspase cascade by caspase 3 leading to apoptosis; B: Mitochondria-dependent extrinsic pathway: Fas-FasL ligation trigger to activate the pro-caspase 8, which cleave Bid (pro-apoptotic Bcl-2 family molecule) to form truncated Bid (tBid). Then, mitochondrial dependent cell death begins with tBid.

II.5.1. Extrinsic pathway

The extrinsic pathway initiates from outside the cell through triggering the activation of transmembrane “death receptors” that are members of the TNF receptor gene superfamily. Members of this receptor family bind to extrinsic ligands known as pro-apoptotic ligands [178] and transduce intracellular signals that ultimately result in the destruction of the cell [179, 180]. To date the best characterized ligands of these receptors are FasL, TNF-α, Apo3L and Apo2L and corresponding receptors are FasR, TNFR1, DR3 and DR4/DR5, respectively [184-186]. The signal transduction of active cell death process involves several caspases. Activated caspases have an effect on several cellular functions as part of the process that results in the death of the cells [178].

The signal transduction of mitochondrial-independent active cell death process involves binding of a pro-apoptotic ligand (such as FasL) with its receptors (Fas) on the surface of a target cell. The cytosolic tail of receptors contains a death domain, which when activated, binds to an adaptor protein, which in turn recruits the specific procaspase-8 and -10 and activates them by proteolytic cleavage [181] that finally initiates the proteolytic caspase cascade leading to apoptosis. Activated caspase 8 triggers the caspase cascade via two different pathways, leading to cell death. In type 1 apoptosis, such as in lymphocytes, caspase 8 activates caspase 3 whereas in type 2 apoptosis, like in hepatocytes and pancreatic cells, caspase 8 activate the pro-apoptotic molecule Bid and go ahead for apoptosis via the disruption of mitochondrial membrane and cytochrome C release [182] (Fig II.8). The T cell death by type

1 and type 2 Fas induced apoptosis fate is decided by the ratio between proteolytically activated effector caspases, X-chromosome linked inhibitor of apoptosis protein and proto-typical effector caspase substrate inhibitor of caspase-activated DNase. Interestingly, HCV specific intrahepatic lymphocytes contribute to bystander killing via Fas-FasL interaction [183], which support the fact that the liver facilitates liver-trapped activated T cell apoptosis [190].

II.5.2. Intrinsic pathway

The intrinsic or mitochondrial pathway is initiated within the cell, involving non-receptor-mediated intracellular signals and inducing activities in the mitochondria that initiate apoptosis. DNA damage, loss of cell-survival factors or other types of severe cell stress causes the induction signal for the intrinsic pathway. This passive death process pivots on the balance of activity between pro- and anti- apoptotic signals of the B cell lymphoma 2 (Bcl-2) family proteins [184]. This balance is maintained by regulation of the permeability of the mitochondrial membrane and by the pro- or anti-apoptotic signal that will be released inside the cell [185]. Following mitochondrial permeabilization, the intrinsic pathway divides into two pathways: Apoptosis protease-activating factor-1 (Apaf-1) dependent and Apaf-1 independent pathway. In Apaf-1 dependent pathway, release of cytochrome c from mitochondria, by triggering the pro-apoptotic Bcl-2 family member [186], and ATP activate monomer inactive Apaf-1 proteins by a conformational change, leading to form a heptamer of Apaf-1 molecules called apoptosome [187].

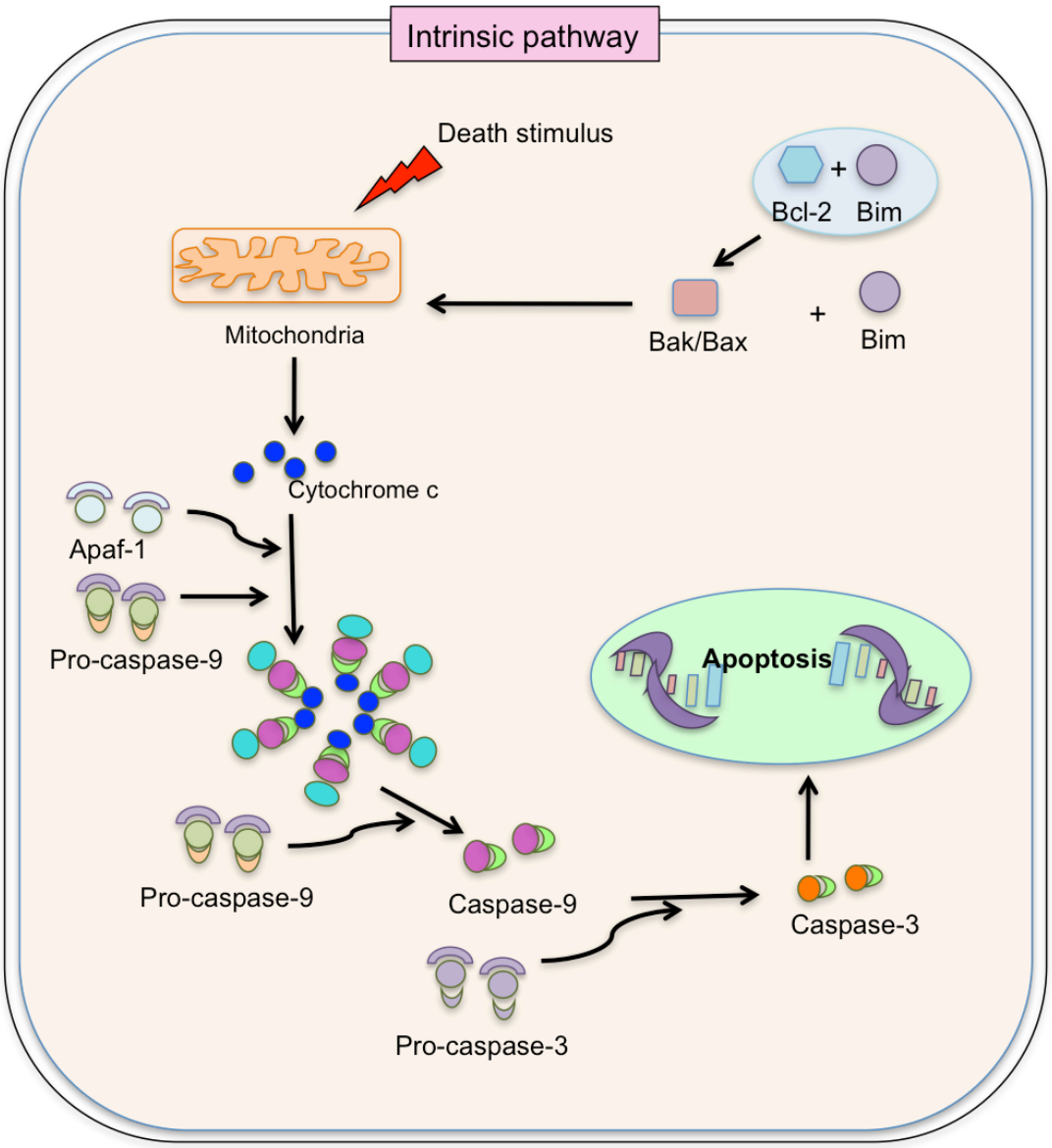


Figure II.9: Apoptosis: Intrinsic pathway. Death stimulation up regulates Bcl-2 interacting mediator leading to the separation from Bcl-2, favoring the activation of Bax, Bak, which form pores in the mitochondrial membrane leading to release of cytochrome c. Cytochrome c with Apaf-1 and procaspase 9 participate in the formation of apoptosome, which activate caspase 9. Caspase-9 activates caspase 3 after cleavage of pro-caspase-3. That caspase-3 triggers to induction of caspase cascade and cell death. Apaf-1: Apoptosis protease-activating factor-1. Bim: Bcl-2 interacting mediator.

Apoptosome allows activation of pro-caspase 9, which consequently triggers the caspase cascade [188]. On the other hand, in Apaf-1 independent pathway, permeabilization of mitochondrial membrane release DIABLO like proteins, which activates effector caspases by provoking inhibitors of apoptosis proteins [189] and triggers caspase cascade [190, 191] (Fig II.9).

The balance of pro- and anti-apoptotic proteins maintains the apoptotic activity [191]. The Bcl-2 family members regulate mostly neglect or intrinsic pathway. This family is subdivided into three groups of proteins on the basis of their functions and the number of Bcl-2 homology (BH) motifs included in their primary structure; first group: “anti-apoptotic multidomain” members, such as Bcl- xL, have four BH domains (BH1 to BH4) which inhibits apoptotic process. Other two groups of “pro-apoptotic multidomain” members, which are Bax-like proteins and “BH3-only” proteins [192]. Bax-like proteins possess three BH domains (BH1 to BH3), including Bax, Bak, and Bok, which are referred as death effector members. BH3-only members contain BH3 domain, including Bim, Bad, Bik, Puma, Noxa and Bid and are known as messengers of death. In addition, C-terminal transmembrane fragment is thought to confer anchorage to mitochondrial membranes, which is also possessed by most multi-BH members and several BH3-only proteins.

Three models (Fig II.10) have been postulated by which the BH3 family promotes passive cell death in which Bax and Bak bind directly or indirectly with cell death sensitizer (e.g., Bad, Bik) and activators of cell death (e.g., Bim, tBid). The direct activation model proposes that sensitizer BH3-only

proteins displace the activator BH3-only proteins from the anti-apoptotic proteins to promote apoptosis. Anti-apoptotic proteins inhibit the activator BH3-only proteins but not Bax and Bak to suppress apoptosis.

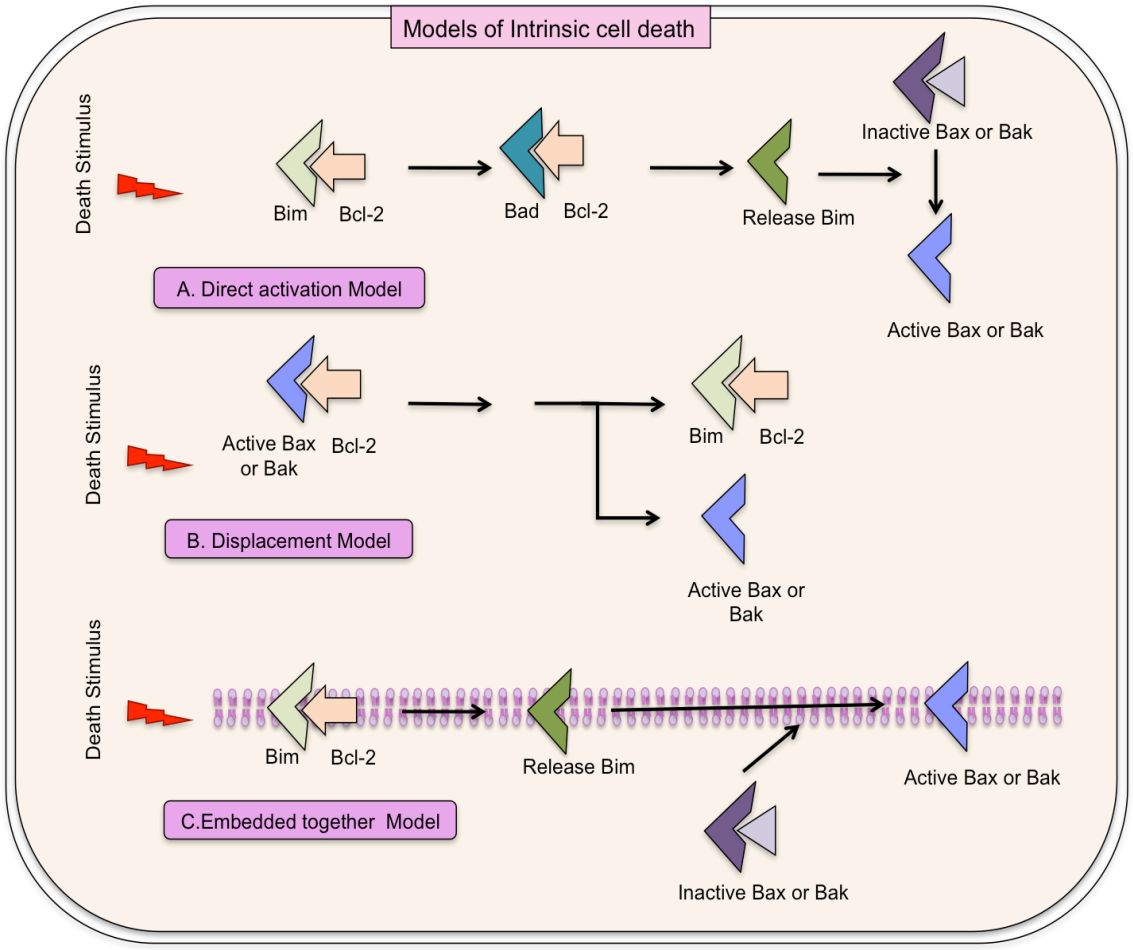


Figure II.10: Models for intrinsic cell death. A: Direct activation model postulates Bcl-2 inter- acting mediator (Bim) is required for activating Bax and Bak. Anti-apoptotic proteins inhibit BH3-only proteins to suppress apoptosis, but not Bax or Bak. Replacement of Bim to sensitizer BH3-proteins from the anti-apoptotic proteins occurs to promote apoptosis; B: The displacement model proposes that anti-apoptotic proteins for cell survival must sequester constitutively active Bax and Bak in cells. Bim inhibits their respective anti-apoptotic proteins by playing sensitizer role to promote apoptosis; C: Embedded together model highlights the active role of the membrane, which is not defined in direct activation model and displacement model. Bcl-2 family proteins insert into and change their conformations that dictate their functions at the membrane. Sensitizer BH3-only proteins relocate the activator BH3-only proteins and Bax/Bak from the anti-apoptotic proteins to endorse apoptosis. Activator BH3-only proteins recruit Bax to the membrane to induce mitochondrial outer membrane permeabilization and apoptosis. These reversible interactions are directed by equilibrium constants that are depended on the concentrations and interactions of the proteins with each other and with membranes.

In the displacement model, Bax and Bak are sequestered by anti-apoptotic proteins for cell survival and constitutively active in cells. BH3-only proteins play the sensitizer role and inhibit their respective anti-apoptotic proteins to promote apoptosis. The third model, called embedded together model, highlights the interactions occurring in and on membranes, which were not explained by direct activation and displacement model. In embedded together model, Bcl-2 family proteins insert into and change their conformations according to their functions in membrane [193]. The predominantly studied messenger death molecule, Bcl-2 interacting protein (Bim) will be focused further.

II.6. BIM

Bim/Bod is a pro-apoptotic protein belonging to the BH3-only group of Bcl-2 family members and is being called the “ghost” molecule or “suicide” molecule, which enables cells to expire gracefully. Two independent studies discovered Bim as a Bcl-2 binding protein and Mcl1- binding protein in 1998 [201, 202]. Bim induces apoptosis by binding to and antagonizing anti-apoptotic members of the Bcl-2 family. The Bim interactions have been observed with Bcl-2 family members, such as Bcl-2, Bcl-xL, Mcl-1, Bcl-w, etc [194, 195].

Bim is well known pivotal initiator of apoptosis in thymocyte-negative selection. Bim has 19 Bim isoforms including three major isoforms, which have distinct sizes and pro-apoptotic activities in the mammals, caused by

alternative splicing: BimEL (extra long), BimL (long) and BimS (small) [73]. The shortest form, BimS, is the most potent and is generally only transiently expressed during apoptosis [196]. The other two isoforms are sequestered to the dynein motor complex, and apoptotic activity of these longer isoforms is regulated by phosphorylation [197, 198], which is triggered by environmental stress, resulting in its dissociation from the dynein complex and increasing apoptotic activity.

Expression of Bim is up regulated in human T cells in response to TCR-triggering by protein kinase C and calcineurin pathways [199]. Nevertheless, there are other mechanisms involved in Bim up-regulation during chronic infection, such as the effect of certain cytokines. In fact, in a persistent viral infection animal model, Bim-mediated apoptosis correlates with low IL-7 receptor expression on specific T cells [200].

The regulation of Bim expression at transcriptional level in growth factor deprivation and in endoplasmic reticulum stress has observed by the class O fork-head box transcription factor (FOX03A) and transcriptional factor CEPB-α respectively [201, 202]. Post-transcriptional phosphorylation of Bim can also regulate its function. Phosphorylated Bim is targeted for proteasomal degradation and avoid its interaction with Bax, thus maintaining cell existence [203, 204]. The signaling adaptor TNFR-associated factor 1 (TRAF1) negatively correlates with Bim and it contributes to CD8 T cell-mediated control of chronic viral infections. In addition, linking between survival effects of TRAF1 and TRAF1-dependent Bim down-modulation has been shown in

CD8 T cells [205-207]. TRAF1 is particularly vanished from virus-specific CD8 T cells during the chronic human immunodeficiency virus and lymphocytic chorio-meningitis virus (LCMV) infection [208].

Bim plays a vital role in the immune system, in bone biology and in tumor-genesis by inducing apoptosis[209]. Bim in T cells, B cells, neurons and many other cell types can trigger apoptosis [209]. Gene targeting in mice for the important region for apoptosis, BH3 region, uncovered the important physiological role in Bim [210]. In fact, in the absence of Bim, leukocytes in blood as well as in LNs, thymus, and spleen were high in number [210]. The role of Bim in apoptosis has been revealed in Bim-/- thymocytes, which were more resistant to apoptosis after different apoptotic treatment such as ionomycin, taxol and γ- irradiation [210].

II.6.1 Death of activated T cells by Bim:

The liver is having a property that might explain its role in inducing tolerance due to its recognition as an alternative primary activation of CD8 T cells site. The phenotype of activated CD8 cells in the liver was the same as in lymph nodes. However, liver-activated CD8 T cells displayed poor effector functions and a unique $CD25^{low}$ $CD54^{low}$ phenotype, which was associated with increased expression of the Bim and caspase-3, demonstrating that these cells are programmed to apoptosis following intrahepatic activation. Strikingly, Bim deficient T cells survived following intrahepatic activation [172]. Therefore, the phenotype and fate of naïve CD8 T cells activated by

hepatocytes in vivo could explain the death penalty role of Bim in chronic hepatotropic viral infection [172]. The distinct phenotype can be due to the lack of co-stimulatory molecule expression on hepatocytes [177]; however the treatment with IL-2 or anti-CD28 antibodies could rescue hepatocyte-activated cells from death [211].

Lymphocyte fate deciding pathways synergize to kill activated T cells in chronic herpes simplex viral immune responses, whereas death of activated T cells in acute immune responses relies only on the mitochondrial pathway involved only Bim with no contribution by Fas, which showed critical overlapping roles for Fas and Bim in T cell death during immune response shutdown, leading to immune tolerance [212].

II.6.2. BIM in Hepatitis:

Bim has been shown to be important for CD8 T cell viability during chronic LCMV infection in mice [213]. In this study, in Bim mutated mice, Bim mutation almost completely blocked the deletion of cognate antigen specific CD8 T cells in liver during chronic viral infection. Bim has a critical role in maintaining naive and memory T cells in LCMV infection [214]. In another study, it has been shown that a defect in apoptosis dramatically not only enhances the antigen-specific memory T cells but also increased the number of virus-specific $CD4^+$ T cells in the lymph nodes following acute LCMV infection, compared to the parental genotypes or wild type mice [215]. Therefore, the loss of both Bim and Fas caused the increase in memory T

cells in acute LCMV infection [215]. The Bim role has been demonstrated in the development of LCMV-induced, T cell-mediated hepatitis by controlling the apoptosis of both T cells and hepatocytes [216].

Bim attrition of virus specific CTLs during HBV infection has also been confirmed [217, 218]. The gene expression profile in HBV infection showed different patterns of gene expression on HBV-specific $CD8^+$ T cells according to viral control. Bim was one of the up-regulated genes in HBV-specific $CD8^+$ T cells from patients with chronic HBV infection. Blocking Bim-mediated apoptosis improved recovery of HBV-specific $CD8^+$ T cell function [217]. Furthermore, the elevated apoptosis has been observed not only with Bim tolerogenic phenotype, but also with co-inhibitory signals through CTLA-4 [218] or T cell-intrinsic transforming growth factor-β [219].

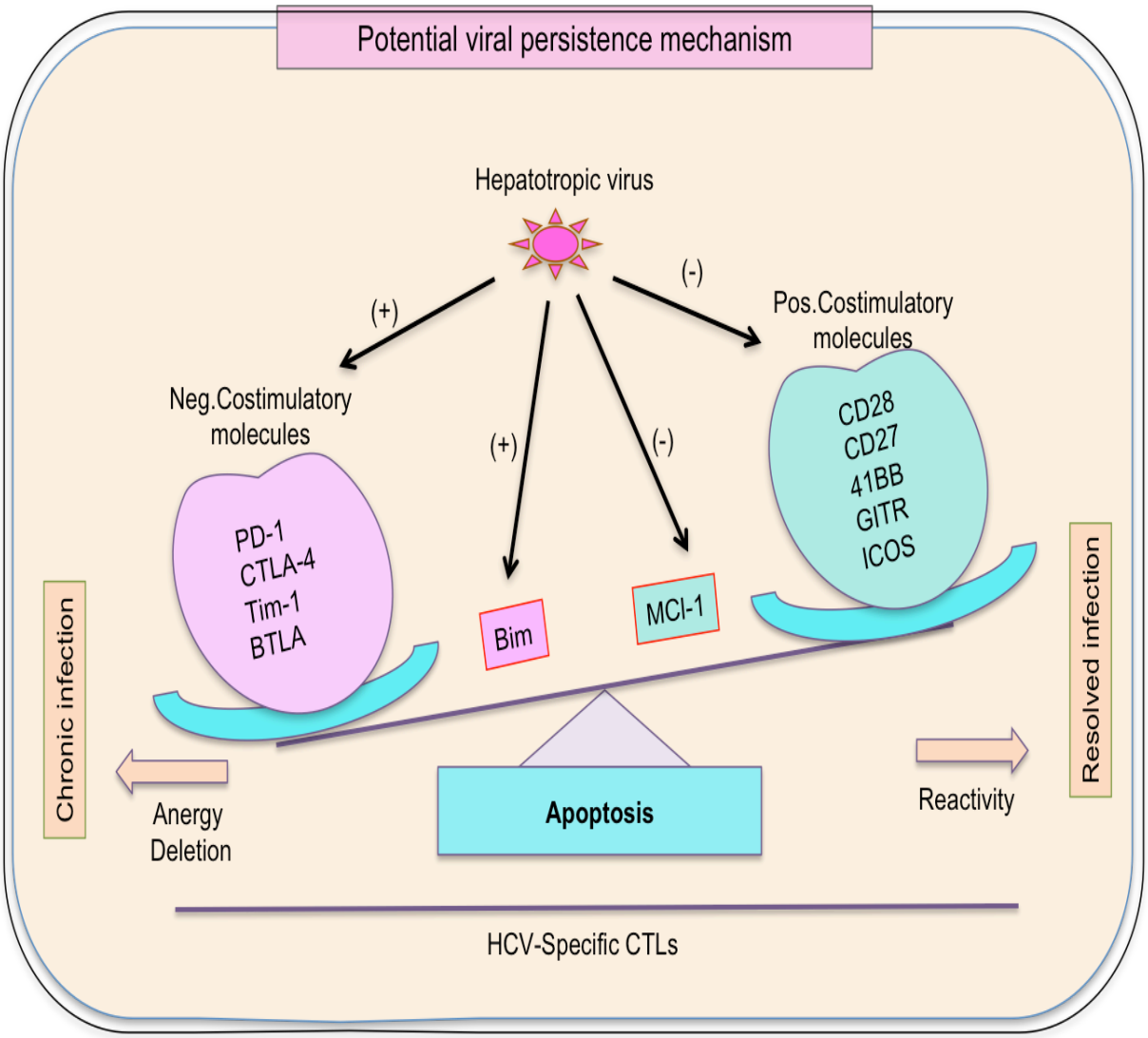


Figure II.11: Balance between co-stimulatory/ apoptotic molecules and viral-specific cyto- toxic T lymphocytes reactivity according to infection outcome. Neg.: Negative; Pos.: Positive; CTLs: Cytotoxic T lymphocytes; ($^{+}$): Possible molecules induced by viral infection; (-): Possible molecules down-regulated by viral infection; BIM: Bcl-2 interacting mediator; Mcl-1: Myeloid cell leukemia sequence-1.

In HCV chronic infection, HCV specific CD8 cells are depleted by Bim mediated attrition, and remaining cells are functionally exhausted. The cell survival factor CD127 counteracts the induction of apoptosis after antigen encounter through myeloid cell leukemia sequence-1 (Mcl-1) expression and Bim down-regulation [220] after the cognate antigen recognition by TCR. In addition, cleavage of Mcl-1 by caspases modifies its subcellular localization, increases its association with Bim and inhibits its anti-apoptotic function [221]. Therefore, Mcl-1 will be discussed further.

II.7 Myeloid cell leukemia-1 (Mcl-1):

Mcl-1 was originally identified as a gene up-regulated early in the differentiation program of the human myeloid leukemia cell line, ML-1 [222]. Mcl-1 is an anti-apoptotic molecule of Bcl-2 family, which promotes cell viability as anti-apoptotic member of this family [223]. The expression of Mcl-1 affects the programming of differentiation or development and cell viability or death. Moreover, according to the data, Mcl-1 may function by providing short-term enhancement of cell viability [221]. In addition, Mcl-1 deficiency results in periimplantation lethality [220] and genetic studies have demonstrated that Mcl-1 functions as an important anti-apoptotic protein in several different cell types. Deletion of Mcl-1 in mice leads to embryonic lethality owing to a failure of implantation of the blastocyst in the uterus [224]. Mcl-1 also promotes the survival of neutrophils and hematopoietic stem cells [225, 226]. Mcl-1 is a highly regulated protein, suggesting that the ability to closely control Mcl-1 gene expression and protein level is critical for the fine

tuning of cell fate decisions, more particularly cell death and survival, but also differentiation.

Mcl-1 is Bcl-2 family member and therefore, shows sequence similarity, particularly in the carboxyl portion, to Bcl-2 [222]. But, it is distinct from Bcl-2 and Bcl-xL because it lacks a true BH4 domain and is a larger protein that encodes an additional internal PEST domain. Mcl-1 is regulated at the transcriptional, post-transcriptional and translational levels. Full-length Mcl-1 consists of three coding exons, a splice variant; Mcl-1S arises by the juxtaposition of exons 1 and 3. Full-length Mcl-1 contains the BH1–BH4 Bcl-2 homology domains while Mcl-1S contains only the BH3 domain [221].

II.7.1 T cell survival by Mcl-1

Bcl-2 family members including Mcl-1 are expressed in T lymphocytes. It was recently uncovered that among signal transduction pathways downstream of TAK1, JNK mediates a survival program through Mcl-1 stabilization downstream of IL-2R in activated T cells and that blockade of TAK1-JNK pathway can eliminate activated T cells by apoptosis [227]. Moreover, IL-7 up-regulates Mcl-1 mRNA expression in T cells and hence, Mcl-1 plays an important role protection of T-cell survival by IL-7, which strongly promotes Mcl-1 stability, possible by controlling lysine-directed ubiquitination [228]. In addition, Mcl-1 is required for survival in developing thymocytes, primary, and mature T lymphocytes [220, 229]. Furthermore, peripheral T cells die upon drug-induced deletion of Mcl-1 in vivo and under different in vitro conditions [220, 230].

II.7.2 Mcl-1 in Hepatitis

Although it is clear that Mcl-1 is an important molecule for T cell survival, aspects of its role in hepatitis remain to be addressed. Mcl-1 as an anti-apoptotic molecule has a pivotal role in impairment of T cell. Hepatitis B virus X protein, which is implicated in pathogenesis of HBV, enhances cisplatin-induced hepatotoxicity via a mechanism involving degradation of Mcl-1 [231], which could lead to the T cell apoptosis. In HCV infection, the core protein is a novel BH3-only viral homologue that contributes to the induction of apoptosis and the over expression of Mcl-1 protects against core-induced apoptosis [232]. During LCMV infection, over expression of Mcl-1 protected activated T cells from death, whereas deletion of Mcl-1 during the course of infection leads to a massive loss of LCMV-specific $CD4^+$ and $CD8^+$ T cells [233]. In contrast, additional loss of Bax and Bak completely rescued Mcl-1-deficient effector T-cell number and function, without enhancing T-cell proliferation. Therefore, Mcl-1 is critical for promoting effector T-cell responses, but does so by combating pro-apoptotic molecules (beyond Bim) [233].

The process of T cell death during chronic viral infection is determined by a carefully balanced and complex group of pro- and anti-apoptotic proteins of the Bcl-2 family, such as Bim and Mcl-1 [234] (Fig I11). Interestingly, persistent hepatotropic viral infection is characterized by continuous TCR triggering and CD127 down-regulation on viral-specific CTLs [235], which could favor Bim up-regulation. CD127 plays an essential role in mature lymphocyte survival by counteracting the induction of apoptosis after antigen

encounter through regulation of some of the B cell lymphoma 2 (Bcl-2) proteins, enhancing IL-2 secretion and life span [236]. Therefore, HCV could modulate CD127 expression on HCV-specific CTLs to impair the quality of the adaptive immune response by IL-7 deprivation, as a survival strategy [121, 166]. Passive T cell death, or death by cytokine deprivation, is controlled by members of the Bcl-2 family, such as Bim and Mcl-1. In addition, it is well known that Bim is clearly involved in intrahepatic specific-CTL apoptosis in animal models [172, 200, 237]. Furthermore, Bim pro-apoptotic effect is blocked by the action of Bcl-2 family anti-apoptotic proteins such as Mcl-1 and Bcl-2 [200, 237]. Interestingly, the co-deletion of Bim fails to prevent the loss of Mcl-1-deficient T cells during LCMV infection [233], clearly pointing out that T cell death also depends on the anti-apoptotic protein expression.

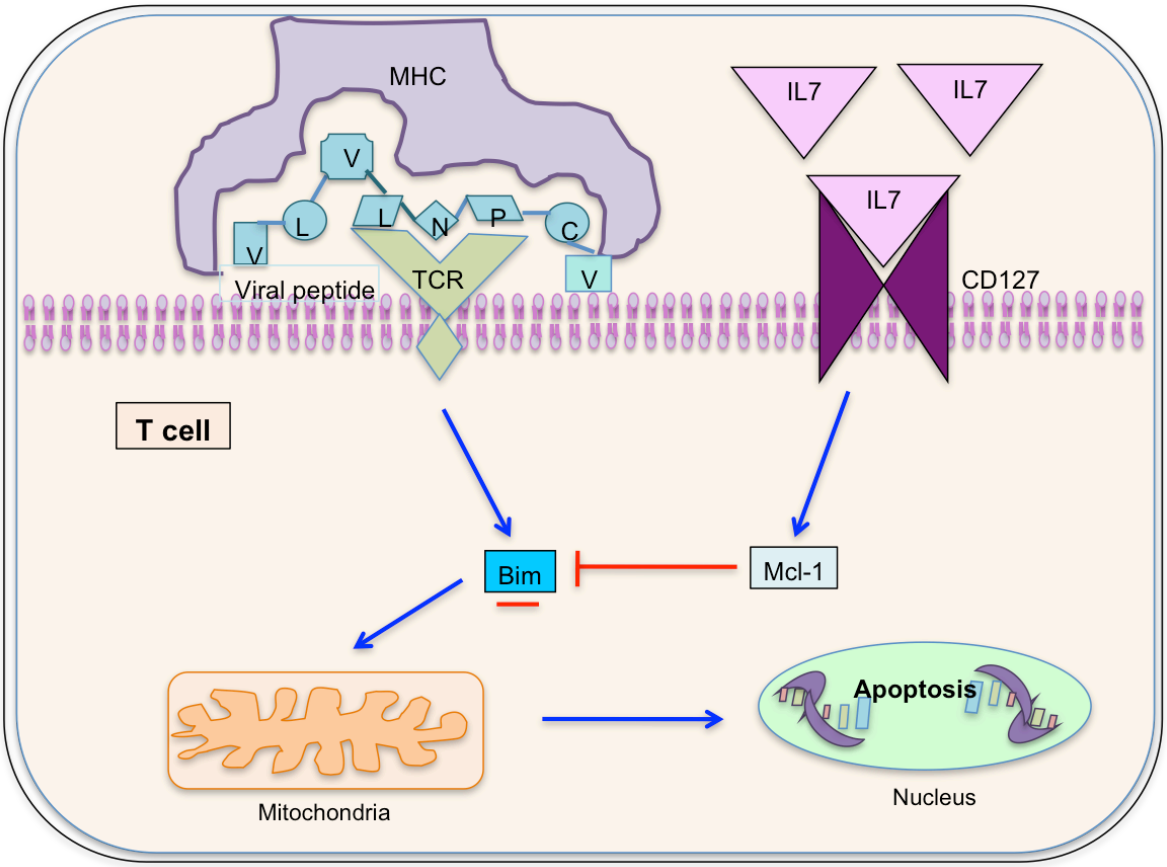


Figure II.12: Cell survival marker CD127 modulates Bim and myeloid cell leukemia sequence-1 expression on hepatitis C virus-specific cytotoxic T lymphocytes after cognate antigen stimulation. Misbalance of Mcl-1/Bcl-2 interacting mediator (Bim) triggers to apoptosis of hepatitis C virus specific cytotoxic T lymphocytes. TCR: T cell receptor; Mcl-1: Myeloid cell leukemia sequence-1.

Bearing in mind all these previous facts, this study tries to test a theoretical model to explain specific CTL deletion during persistent hepatotropic viral infection (Fig I.12). This model hypothesizes that CD127 phenotype modulates Bim and Mcl-1 expression on virus-specific CTLs, leading to Mcl-1/Bim imbalance during persistent infection, which impairs T cell reactivity through apotosis induction and suggesting that restoration of T cell function could occur by correcting the levels of Mcl-1 and Bim expression. Therefore, in this study, the correlation between CD127 expression on HCV-specific CTLs and the Mcl-1/Bim phenotype and their effect on T cell reactivity were analyzed.

III

HYPOTHESIS

AND

OBJECTIVES

III. Hypothesis and Objectives:

Based on the rationale about apoptotic molecules and the impairment of HCV-specific CTL response during chronic hepatitis C infection discussed in previous chapter, we propose the following hypothesis:

Hypothesis:

HCV could modulate CD127 expression on HCV-specific CTLs in order to promote a pro-apoptotic state due to a misbalance between pro- (Bim) and anti-apoptotic (Mcl-1) molecules that could alter the ability of T cell response to control viral infection. Modulation of these pathways could restore the HCV-specific cytotoxic response in chronic patients characterized by low CD127 expression.

To test this hypothesis, we set out the following study objectives:

General objective:

To correlate the functional features and the apoptotic phenotype (Bim/Mcl-1) of specific cytotoxic response against HCV depending on the CD127 expression level.

Specific objectives:

- To correlate CD127 expression level with viral replication and liver damage on HCV specific T cells

- To analyze the frequency of HCV-specific cytotoxic T cells in peripheral blood according to CD127 expression level.

- To study the phenotypic and functional characteristics of HCV-specific cytotoxic T cells in peripheral blood according to CD127 expression level:

  - Comparison of the proliferation capacity after specific stimulation with presence or absence of apoptosis inhibitor according to the CD127 expression level.

  - Production of interferon gamma according to CD127 expression level.

  - Bim and Mcl-1 phenotype, directly *ex vivo* and after specific *in vitro* stimulation in presence or absence of apoptosis inhibitor, according to CD127 expression level, and correlation of Bim/Mcl-1 balance with HCV-specific T cells reactivity.

IV

DESIGN, MATERIAL AND METHODS

IV. Design, Material and Methods

IV.1 Study Design:

IV.1.1 Type of study

To test the hypothesis, we designed a non-experimental, analytical, cross-sectional study, which analyzed various characteristics of the HCV-specific cytotoxic cellular response such as frequency, clonal expansion capability, ability to produce IFN-γ, phenotype associated with apoptosis (Bim/Mcl-1), depending on the expression level of the survival marker CD127. Previously we have shown that in subjects with resolved HCV infection, $CD127^{high}$ expressing HCV-specific T cells are predominant while in chronic patients these cells show a $CD127^{low}$ phenotype [174].

IV.1.2 Selection of study subjects:

The study was conducted within the health area of Guadalajara University Hospital, University of Alcalá, Spain. Samples were collected from subjects with HCV infection who referred to the Translational Hepatology Unit, Gastroenterology Department of the Guadalajara University Hospital, (University of Alcalá, Spain). The study was carried out between January 2007 and August 2011.

1. Admission criteria in the study:

During the study period, the patients diagnosed with HCV infection, were included by consecutive sampling after being referred to the Department of Gastroenterology and if they met the following inclusion and exclusion criteria:

1.1 Inclusion criteria:

- Age between 18 and 65.

- Positive serology for HCV infection.

- HCV patient with HLA-A2 positive haplotype. The study was restricted to this group only because the method used to detect HCV specific CD8 lymphocytes was able to visualize HLA-A2 restricted HCV-epitopes.

- Genotype 1: HLA-$A2^{+}$ HCV infected patients with genotype 1, because the immunodominant HCV epitopes analyzed in the study belong to HCV genotype 1.

- HLA-$A2^{+}$ HCV infected patients with detectable HCV specific CTLs directly *ex vivo*: HCV infected cases, who have detectable HCV specific CTLs against HCV specific epitopes $NS3^{1406–1415}$ and $NS3^{1073–1081}$ were included in this study.

- ❖ Signed an informed consent with the agreement to participate in the study.

1.2 Exclusion criteria:

- ❖ HCV genotype other than genotype 1.

- ❖ Consumption more than 40 grams of ethanol per day for men and 20 grams for women.

- ❖ Presence of other causes of chronic liver diseases such as hemochromatosis, autoimmune hepatitis, Wilson's disease, primary biliary cirrhosis, porphyria cutanea tarda, non alchoholic steatohepatitis and HBV co-infection.

- ❖ Primary or secondary immuno-suppression: The subjects with primary and secondary immuno-suppression were excluded from the study to avoid the possible consequences of immuno-suppression on the cytotoxic response against the virus. Therefore, HIV-infected subjects and patients with immunosuppressive treatments were excluded.

- ❖ HLA-$A2^{+}$ HCV infected patients with no detectable HCV specific CTLs directly *ex vivo*: HCV infected cases, who do not have detectable

HCV specific CTLs against HCV specific epitopes $NS3_{1406–1415}$ and $NS3_{1073–1081}$ were excluded in this study.

- ❖ Patients, who were not ready for signing an informed consent with the agreement to participate in the study, were excluded.

IV.1.4 Study Variables:

To carry out our objectives previously described, we defined two different studies, one analytical cross-sectional study and another quasi experimental type pre-post study. In the following lines are presented the variables included in both studies:

IV.1.4.1 Analytical cross sectional study.

1. Independent variable:

The IL-7 receptor (IL-7R) CD127 as a survival marker is important for the establishment and maintenance of the cytotoxic T lymphocytes in viral infection. Therefore, it is supposed that there could be certain molecules in HCV infection that could change in cytotoxic T cells according to CD127 expression and lead to death of the HCV-specific T cells. We have previously shown that CD127 expression on HCV specific CTLs could decide outcome of HCV infection [174]. $CD127^+$ expression was found in most sustained virologic responder and $CD127^-$ phenotype was observed in chronic HCV infection.

Therefore, in this study, the independent variable is dichotomous categorical variable with two groups depending upon CD127 expression level ($CD127^{low}$ and $CD127^{high}$) and considered CD127 ^{high} or CD127 ^{low} according to our previous work. Level of CD127 expression was decided by the Mean fluorescence intensity (MFI) after anti-CD127-FITC staining of HCV specific CTLs.

- **$CD127^{low}$:** $HLA\text{-}A2^{+}$ HCV cases with directly *ex vivo* detectable HCV specific CTLs, which were showing less than 20 MFI for CD127-FITC on $pentamer^{+}/CD8^{+}$ cells.

- **$CD127^{high}$:** $CD127^{high}$ expression level was considered, within $HLA\text{-}A2^{+}$ HCV cases with directly *ex vivo* detectable HCV specific CTLs with more than 20 MFI for CD127-FITC on $pentamer^{+}/CD8^{+}$ cells.

2. Dependent Variables:

To analyze the quantitative and qualitative (function and apoptotic phenotype) characteristics of the HCV-specific cytotoxic response according to CD127 expression, the cytotoxic response against two immunodominant HLA-A2 restricted HCV-epitopes were chosen ($NS3_{1406\text{-}1415}$, and $NS3_{1073\text{-}1081}$). To test these features the following variables were recorded:

- Frequency of HCV specific $CD8^+$ T cells out of total $CD8^+$ T cells: The frequency of HCV specific CTLs was analyzed out of total $CD8^+$ T cells from selected HCV populations and recorded as percentage after staining with anti-CD8-mAbs and pentamer. It is a continuous quantitative variable with a range between 0-100%.

- Proliferation of HCV specific $CD8^+$ T cells after specific stimulation in presence or absence of z-VAD-fmk: The ability of HCV specific CTLs to proliferate was analyzed after 10 days specific stimulation with or without pan-caspase inhibitor z-VAD-fmk. It is a categorical variable with two categories in each kind of experiment (proliferation positive or negative).

- Serum Alanine amino-transferase (ALT) in international units per liter (IU/L): ALT was used as an index of the degree of liver inflammation. It is a continuous quantitative variable with a range from 0 to 170 IU/ml. ALT>40 IU/mL was considered high while ALT<40 IU/mL was considered low.

- Virological status: Serum HCV RNA measured in IU/mL and used as an index of degree of viral control. It is a continuous quantitative variable with a range between 1.6×10^1 to 6.06×10^6 IU/mL.

Diagnosis of HCV infected cases was carried out and grouped in sustained virologic responder (SVR) and chronic HCV infection (CHC). Subjects, who have persistent HCV RNA positive in serum for more than 6 months are called as CHC, while subjects who have negative HCV RNA in serum 6 months after finishing HCV treatment are considered as SVR. This outcome of HCV infection correlates with CD127 expression level and is also related to the ALT and viral load levels [174]. Most of the $CD127^{high}$ expressing cases were diagnosed as SVR, in which ALT and HCV RNA level were low, while $CD127^{low}$ expressing HCV specific CTLs were observed in CHC, where ALT and HCV RNA level were high, as it was previously shown in our work [174]. However, there were some cases, which were showing $CD127^{high}$ expression level with diagnosis of CHC. It probably was because of the presence of epitope mutations at TCR interaction sites i.e. escape mutation as it was shown in our previous work [174].

- Production of type I cytokines by HCV specific $CD8^+$ T cells: The HCV-specific CTLs were analyzed for IFN-γ production directly *ex vivo* as percentage of IFN-γ producing HCV specific cells according to CD127 expression level out of total HCV-specific CTLs. It is a continuous quantitative variable with a range from 0-100%.

- ❖ Expression of Mcl-1 and Bim on HCV specific $CD8^+$ T cells: This variable was recorded as MFI after staining with respective fluorescence labeled antibodies. It is a continuous quantitative variable with a range from 0 to 10,000 fluorescence units.

3. Covariates:

The following variables were collected to control possible bias according to the result of the independent variables.

- ❖ Age in years: It is a continuous quantitative variable with range from 18 to 65 years. Age can correlate to the evolution time of chronic hepatitis infection. Furthermore, aged patients show alterations in quality and intensity of the immune system that can affect the study's conclusions.

- ❖ Sex: It is a dichotomous categorical variable. Male gender is associated with a worse prognosis of HCV infection.

- ❖ Time of evolution: It is a continuous quantitative variable with range from 25 to 30 years. Time of evolution was generated by estimating the date on which the person became infected. It is considered that subjects with a longer history of the disease may have a poorer specific immune response.

- HCV genotype (1a and 1b): In this study, to visualize HCV-specific CTLs, we used labeled HLA-class I/HCV-peptide multimers (pentamers). These pentamers were loaded with HCV genotype 1 specific epitope. Therefore, to study the HCV specific CTLs, HCV genotype 1 cases were selected.

- HLA-$A2^{+}$ Haplotype in HCV infected patients: The molecules used to visualize HCV specific CTLs called pentamers, were capable to recognize those cells in only HLA-A2 restricted HCV subjects. Therefore, this variable was used to select the HLA-A2 restricted patients.

IV.1.4.2 Quasi experimental Study:

In this quasi-experimental type of study type pre-post testing dynamics of Bim/Mcl-1/CD127 (dependent variable) after treatment with or without blocking of apoptosis with z-VAD-fmk (independent variable) were analyzed.

1. Independent variable:

The independent variable is a dichotomous categorical one with two possible values depending upon the treatment or not with pancaspase inhibitor z-VAD-fmk to inhibit apoptosis on HCV-specific CTLs after specific in vitro challenge. It was considered positive when z-VAD-fmk was added or negative when β-galactosidase was added as control.

2. Dependent Variables:

The quantitative and qualitative (apoptotic phenotype) characteristics of the HCV-specific cytotoxic response, directly ex-vivo and after specific antigenic stimulation for 10 days, in presence or absence of pan-caspase inhibitor (z-VAD-fmk) were analyzed. To test these features the following variables were recorded:

- Expression of Mcl-1 on HCV specific $CD8^+$ T cells directly ex-vivo and after HCV specific $CD8^+$ T cells specific stimulation in presence or absence of z-VAD-fmk. This variable was recorded as MFI after staining with respective fluorescence labeled antibodies. It is a continuous quantitative variable with a range from 0 to 10,000 fluorescence units

- Expression of Bim on HCV specific $CD8^+$ T cells directly ex-vivo and after HCV specific $CD8^+$ T cells specific stimulation in presence or absence of z-VAD-fmk. This variable was recorded as MFI after staining with respective fluorescence labeled antibodies. It is a continuous quantitative variable with a range from 0 to 10,000 fluorescence units.

- Expression of CD127 on HCV specific $CD8^+$ T cells directly ex-vivo and after HCV specific $CD8^+$ T cells specific stimulation

in presence or absence of z-VAD-fmk. This variable was recorded as MFI after staining with respective fluorescence labeled antibodies. It is a continuous quantitative variable with a range from 0 to 10,000 fluorescence units.

IV.3 Subjects, Material And Experimental Methods

Between January 2007 and August 2011, 24 subjects with HLA-$A2^+$ HCV infection were enrolled in the Translational Hepatology Unit, from the University Hospital of Guadalajara, Spain.

IV.3.1 Samples selected for the study:

The HLA-$A2^+$ HCV infected subjects, who could fulfill the inclusion and exclusion criteria were analyzed for HCV specific CTLs. In this cross-sectional study, peripheral blood mononuclear cells (PBMC) from 24 consecutive HLA-$A2^+$ HCV^+ genotype-1 subjects, who have directly *ex vivo* HCV- specific CTLs against one or two different HCV-NS3 immunodominant epitopes, were obtained. From these cases, 33 different HCV-specific CTL populations were isolated, 20 of them specific against $NS3_{1406–1415}$ and another 13 specific against $NS3_{1073–1081}$. These samples were split into two groups according to CD127 expression level on HCV-specific CTLs. The patients' clinical and virological features, regarding these two CD127 groups, are shown in Table IV.1. CD127 expression levels were analyzed on positive HCV specific CTLs and CD127 expression was considered high when MFI was higher than 20

units.

IV.3.2 Sample and clinical data collection:

All subjects included in this study were subjected to a standardized questionnaire to collect clinical data of interest as described in the study variables section.

1. Virological assessment:

The collected patients went through the virological assessment to fulfill the criteria to include or exclude in the study. Anti-HCV antibodies were determined by enzyme-linked immunosorbent assay performed as per the manufacturer's instructions on the Ortho HCV Version 3.0 ELISA Test (Ortho Diagnostic System, Raritan, NJ). The viral load of HCV was measured by quantitative real-time PCR assay using a Roche kit (Cobas Amplicor HCV Monitor 2.0 Roche, Indianapolis, IN, USA), which has lower detection limit about 40 IU/mL. The HCV genotype was determined by reverse hybridization method and with Inno-LiPPA HCV II, assay kit (Innogentics Inc, Alpharetta, GA, USA). ALT level of patients' serum was determined by using standard biochemical procedures. These tests were conducted in the Laboratory of Virology and Clinical Biochemistry, Guadalajara University Hospital.

Table IV.1. Clinical and virological features of the HCV-specific CTL samples tested in the study.

	CD127 high N=14	CD127 low N=19	P value*
Age (years old)	43 (4)	46 (15)	0.014
Sex (M/F) (%)	78/22	77/23	NS
Diagnosis (%)			
- SVR	86%	5%	<0.001
- CHC	14%	95%	
Time of evolution (years)	24 (6)	29 (24)	NS
ALT (IU/mL)	40 (31)	72 (82)	<0.001
HCV viral load (IU/mL) (log scale)	1.6 (0.8)	6.06 (0.96)	<0.001
Genotype 1a/1b (%)	30/70	55/45	NS
Epitopes tested ($NS3_{1406}$/$NS3_{1073}$) (%)	73/26	54/46	NS

CHC: chronic hepatitis C, CTL: cytotoxic T lymphocyte, F: female, HCV: hepatitis C virus, M: male, N: number of samples, NS: non-significant, SVR: sustained virologic response, *Mann-Whitney U test.

2. Synthetic peptides and Pentamers:

A2-restricted peptides corresponding to the genotype-1 $NS3_{1406–1415}$ region (KLVALGINAV), $NS3_{1073–1081}$ region (CINGVCWTV) and Phycoerithrin-conjugated (PE-conjugated) HLA-A2 pentameric complexes (pentamer) loaded with the same two NS3 peptides were purchased from ProImmune (Oxford, UK).

3. Tissue typing:

To assess the HLA-A2 haplotype of the HCV infected subjects to include in the study, the following protocol was used. In brief, 100uL of peripheral blood was stained with mouse anti-human-HLA-A2 monoclonal antibody labeled with Fluorescein isothiocyanate (FITC) (BD Biosciences, San Diego, CA), mixed properly by vortexing and incubated for 20 minutes at room temperature (RT). At the end of the incubation period, 2 mL of lysis solution was added (BD Biosciences, San Diego, CA) and incubated for another 10 minutes. Finally, the stained blood was centrifuged at 2,000 rpm after vortexing. The obtained pellet was washed twice with wash buffer (PBS^{+} 0.01% FCS) and subsequently analyzed by flow cytometry i.e. FACS calibur cytometer with CELLQuest software (Becton Dickinson, CA). The haplotype HLA-A2 positive was set to a value greater than MFI calculated as the mean of the 10 previously analyzed negative controls (Fig. IV.1).

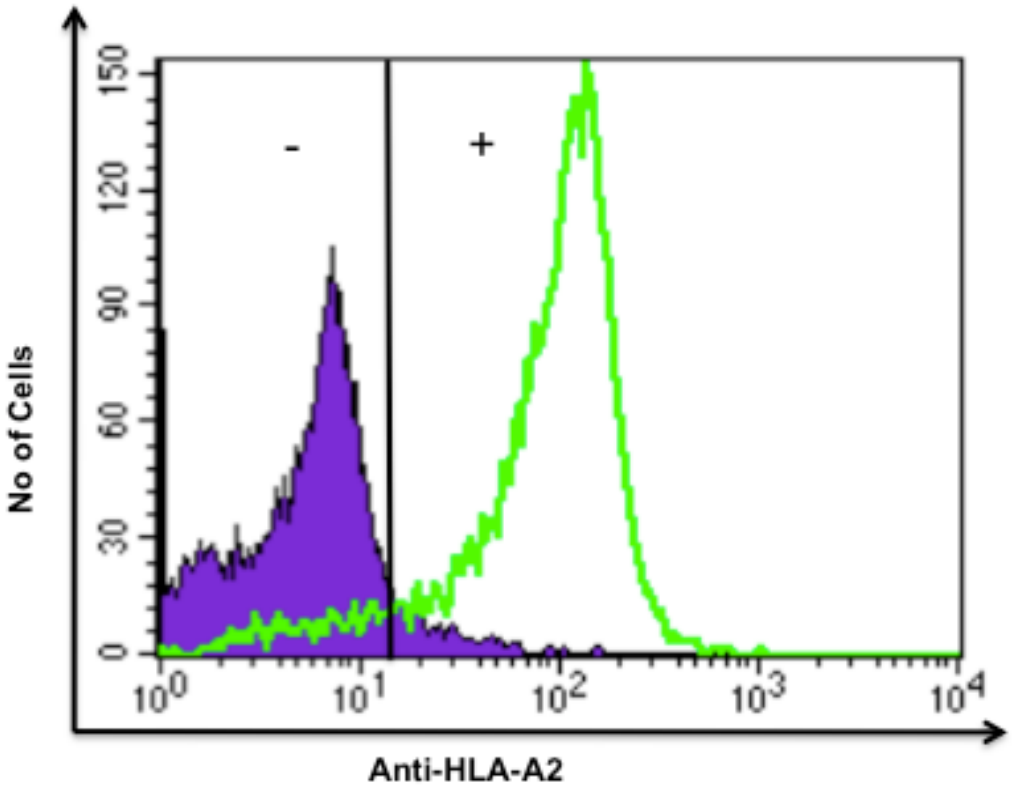


Figure IV.1. Histogram of a HLA-A2 negative and positive sample: The latter peak shows a fluorescence intensity of PBMCs stained with anti-HLA-A2 FITC antibody. More than 10 units of fluorescence in PBMC is HLA-A2$^+$. (-) Negative. ($^+$) Positive.

IV.4 Processing of peripheral blood samples:

For the study, 60 ml of heparinized blood was drawn with anti-coagulant using standard venipuncture technique. The separation of PBMC from the collected blood was done by Ficoll-hypaque method i.e density gradient centrifugation technique. Briefly in a 50 mL Falcon tube, 1/3 of Ficoll-hypaque (Amersham Pharmacia Biotech AB, Uppsala, Sweden) was taken and 2/3 of heparinized blood was gently added from the sidewall of the tube in order to prevent mixing of two layers. The tube was centrifuged at 2,000 rpm for 30

minutes without break. Then, the PBMCs were collected from the interphase of PBS/ficoll with a transfer pipette in a new 15 ml Falcon tube. The collected PBMCs were washed 2 times with Roswell Park Memorial Institute 1640 (RPMI 1640) (Sigma-Aldrich Inc., St. Louis, MO, USA), which was supplemented with 10% fetal calf serum (FCS) (Biochrom AG, Berlin, Germany). Cells were counted in a Neubauer chamber under an optical microscope after staining with Trypan blue for viability of the cells.

On an average, from 60 ml of blood, between 30-40x10^6 PBMCs were obtained. The cells were re-suspended in culture medium i.e. RPMI plus 10% FCS to be used further for analysis of the frequency and phenotype of HCV-specific $CD8^+$ cells and the production of cell lines through specific stimulation with peptides.

1. Pentamer$^+$/$CD8^+$ cell quantification:

For the detection of HCV-specific $CD8^+$ T cells, a technology that enables its count directly ex-vivo without functional assays was used [100, 238, 239]. This technology involves the use of a pentameric molecule (pentamer) which is formed by the union of five HLA molecules of class I loaded with HCV specific peptide linked to a fluorescent molecule, phycoerythrin (PE). This molecule allows specific binding to HCV specific cytotoxic cells and these cells were consequently detected by the fluorescence emission through Flow Cytometry (Fig.IV.2). This technique is

exceptionally antigen-specific and highly sensitive, since it allows recognizing about one antigen specific $CD8^+$ T cell out of 5,000 $CD8^+$ lymphocytes.

To carry out this test, PBMC were purified from heparinized blood samples according to the method described previously. To quantify HCV-specific $CD8^+$ cells, 0.5-1x10^6 PBMC were incubated for 10 min at 37^{o}C with 1 µg of PE-labeled pentamers in RPMI 1640 plus 10% FCS. Cells were washed in PBS and then incubated at 4^{o}C for 20 min with saturating concentrations of directly conjugated anti-CD8-Pe-Cy5 (Cy) mAbs, (Sigma-Aldrich Inc, St. Louis, MO). Subsequently, stained cells were analyzed after washing on a Becton Dickinson FACS® using CELLQuestTM software. The cut-off for pentamer assay was determined by staining peripheral blood mononuclear cells from 10 HLA-$A2^-$ chronically infected HCV patients. Using the mean plus 2 standard deviations as the cut-off point, the lowest percentage at which pentamer-binding $CD8^+$ cells could be detect as a separate cell population was 0.018% out of total $CD8^+$ cells. At least 250,000 PBMCs were analyzed for each staining to achieve a minimum of 50,000 number of $CD8^+$ T cells.

In this study, we worked with two-pentameric complexes whose structure includes two HLA-A2 restricted immunodominant HCV specific peptides. Both of them belong to the NS3 region ($NS3_{1406-1415}$ and $NS3_{1073-1081}$ fragments) [240, 241].

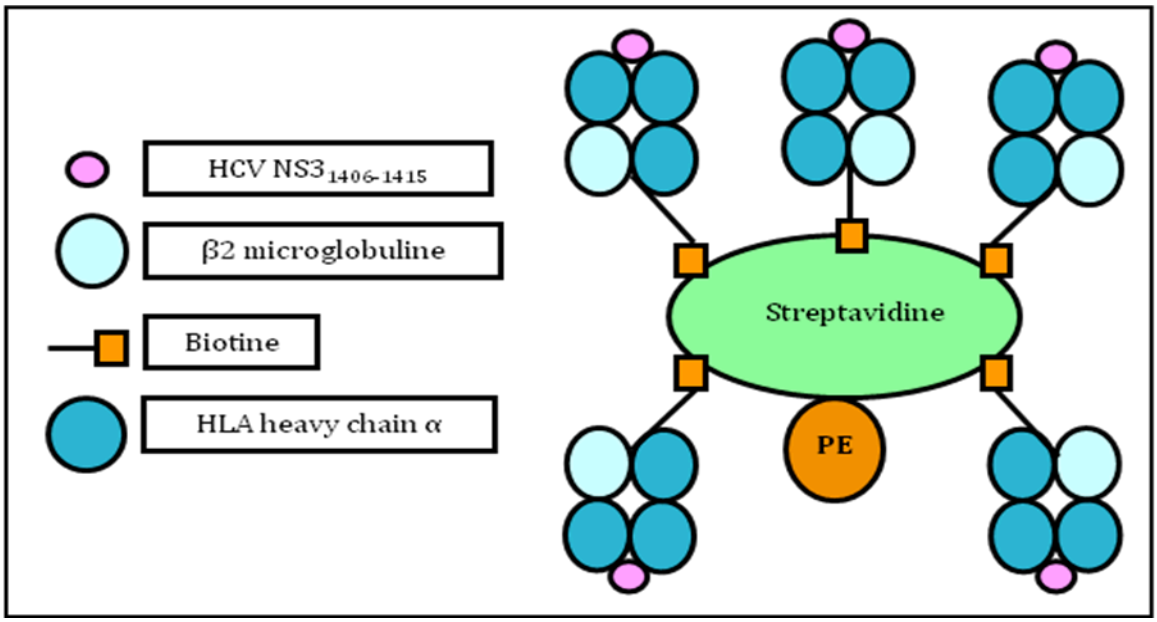


Figure IV.2: Outline of HLA-A2 / peptide pentameric complex $NS3_{1406-1415}$.
PE: Phycoerythrin.

2. Production of T cell lines

To assess the ability of clonal expansion of HCV specific $CD8^+$ T cells in presence of HCV-specific antigen, *in vitro* PBMCs were stimulated with specific epitopes corresponding to the same peptide regions loaded on pentamers ($NS3_{1406-1415}$, and $NS3_{1073-1081}$) (Proimmune Limited, Oxford, UK). Peripheral blood lymphocytes were re-suspended at a concentration of 1 X 10^6/mL in complete medium plus 10% FCS (Table IV.2). To carry out the stimulation, specific peptides were added at a final concentration of 1μM with 25 IU/ml of IL-2 (RD systems) along with tetanus toxoid to a final concentration of 0.5μM. The suspension was distributed carefully without vigorous handling in 96 round bottom well plate and incubated for 10 days at 37°C and atmosphere of 5% CO_2. Here, the tetanus toxoid is used to activate

T helper cells in order to enhance an environment of type-1 cytokines to help in the HCV specific $CD8^+$ T cell proliferation. The recombinant IL-2 (25 IU/mL) (RD Systems) was added at the beginning and the day 3, 5, 7, 9 of culture to allow the cell proliferation. The IL-2 is the third signal necessary for T cell activation, in addition to TCR triggering and positive co-stimulation. These two last signals induce IL-2 receptor expression (CD25) about 48 hours after the interaction between the TCR and the HLA-I/epitope complex. PBMCs were also cultured in the presence of either the pancaspase inhibitor zVAD-fmk (50 ml) (BD Bioscience, San Jose, CA, USA), or β-galactosidase (2 ug/mL) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as control. All cultured cells were harvested after ten days and were analyzed by flow cytometry for pentameric complexes and anti-CD8 antibodies staining as previously discussed.

Table IV.2. Mediums and Buffers used in Experiments:

SOLUTIONS	COMPOSITION
Washing medium for separation of PBMCs	▪ RPMI 1640 medium ▪ 10 % FCS
Washing buffer for PBMCs staining with pentameric complexes	▪ 0.1% Sodium Azide ▪ 0.1% FCS ▪ PBS
Cell Culture Media	▪ RPMI 1640 Medium: 500 mL ▪ Hepes 20 Mm ▪ Sodium pyruvate 0.5 mM ▪ Penicillium100 U/mL ▪ Streptomycin 100 µg/mL ▪ 2 Mercaptoethanol 50 µM ▪ L-glutamine ▪ Essencial amino acids solution 50 X (1/50) ▪ Non-essential amino acids solutions 100 X (1/100) ▪ 10% FCS

PBMCs: peripheral blood mononuclear cells. FCS: fetal calf serum. PBS: phosphate buffered saline. HCl: Hydrochloric Acid. KCl: Potassium chloride.

3. CD127 phenotypic analysis on $CD8^+$/$Pentamer^+$ cells:

Approximately 1 to 1.5x10^6 PBMCs or a variable number of cells were incubated for 10 minutes with 1μM of HLA-A2/peptide pentameric complexes (PE) in RPMI with 10% FCS (Table 2.2) at room temperature and subsequently washed with PBS (phosphate buffer saline) with 0.01% FCS at 2000 RPM for 5 minutes. After washing, cells were incubated with saturating concentrations of anti-CD8-phycoerythrin Cyanine-5 (Pe-Cy5) labeled Abs (R & D Systems Inc., Minneapolis, MN) for 20 minutes at 4^{o}C. Phenotypic analysis was performed only in cases in which more than 10 cells out of 50,000 specific $CD8^+$ T cells were detected directly *ex vivo* or 20 cells out of 50,000 specific $CD8^+$ T cells were visualized after specific *in vitro* proliferation. The positive pentameric staining tubes further proceed for the CD127 staining with 2μL of anti-CD127 antibody (BD Pharmingen, San Jose, CA). After further washing, the stained cell suspension was analyzed by the flow cytometer. Isotype matched control mAbs were used to set the markers.

4. Intracellular Bim/Mcl-1 staining:

To evaluate the expression of the pro-apoptotic molecule Bim and anti-apoptotic molecule Mcl-1 directly *ex vivo* and after antigenic expansion in the presence of z-VAD-fmk or β-galactosidase, HCV specific $CD8^+$ T cells were labeled with pentameric complex-PE and anti-CD8-Cy5 antibody, as described above. Then, the cells were fixed and permeabilized for 20 minutes with Citofix / Cytoperm (BD Bioscience, San Jose, CA). Then the cells stained

with Bim unconjugated rabbit anti-human polyclonal antibody (Cell Signalling Technology, Beverly, MA), Mcl-1 unconjugated rabbit anti-human mAbs (Epitomics, Burlingame, CA, USA) and its isotype-matched control for 45 minutes in respective tubes and goat anti-rabbit IgG2 Alexa Fluor 488 secondary antibody (Invitrogen, Carlsbad, CA) for 30 minutes. Finally, the cells were washed twice with perm wash buffer and analyzed by flow cytometry. Data were expressed as Alexa-Fluor 488 MFI on gated pentamer^{+}/CD8^{+} cells. The absolute value of Alexa-Fluor 488 MFI for Mcl-1 and Bim staining and the difference between these two values were compared according to CD127 expression level, and correlated with pentamer^{+}/CD8^{+} cell reactivity. The cut-off for Mcl-1/Bim positive staining was set according to the mean plus 2 standard deviations of the MFI observed with the matched control staining.

5. Production of interferon-γ:

To test the ability of IFN-γ production, 1x10^{6}/ml PBMCs in RPMI 1640 supplemented with 10% FCS, were stimulated with 2μM HCV specific peptide (eg. $NS3_{1406\text{-}1415}$) for 6 hours at 37°C, in the presence of 10 μg/ml Brefeldin A (Sigma-Aldrich Inc., St. Louis, MO) for the last 4 hours. After washing, cells were labeled with pentameric complex (HLA-A2 / $NS3_{1406\text{-}1415}$) and anti-CD8-PeCy5 antibody as discussed above. After incubation and washing again, the cells were fixed and permeabilized with 250μL of Cytofix / Cytoperm (BD Biosciences, San Diego, CA) for 20 minutes at 4°C. After washing, the cell suspension was added 7μL of anti-IFN-γ conjugated with FITC (BD

Pharmingen, San Jose, CA) and its isotype-matched control (Pharmingen BD, San Jose, CA) and incubated for 45 minutes at RT. Finally, the cells were washed twice and analyzed by Flow Cytometry.

6. Statistical Analysis:

Non-parametric statistical analysis was carried out. Continuous and categorical variables were summarized as median plus interquartile range (IQR) and as frequency distribution respectively. Mann-Whitney-U, Wilconxon and Spearman tests were employed where appropriate. All the tests were two tailed and with a significance level of $p<0.05$.

V

RESULTS

V. Results

In persistent HCV infection, HCV specific CTL reactivity is impaired and thus leading to chronic HCV infection. The regulation of CTL reactivity could be affected by CD127 (IL-7-R) expression on CTLs through Mcl-1/Bim balance modulation. Bim, as a pro-apoptotic molecule, is blocked by the action of anti-apoptotic molecule- Mcl-1. Correlation of Mcl-1 and Bim expression ratio with T-cell reactivity on HCV specific CTLs can help for better understanding of the mechanism involved on HCV-specific CTL hyporeactivity during chronic HCV infection. Therefore, the aim of this study was to analyze the correlation of the Mcl-1/Bim phenotype with their effect on T cell reactivity according to CD127 expression on HCV-specific CTLs. As previously commented, it has been already shown a negative correlation between CD127 expression level and HCV control [174].

In this study, we obtained blood from HCV^+ $HLA\text{-}A2^+$ genotype 1 patients. Peripheral blood lymphocytes were isolated from these patients and HCV-specific CTLs were visualized by staining with anti-CD8 and HLA-A2/peptide pentameric complexes (Pentamer). Furthermore, Mcl-1/Bim/CD127 phenotype of HCV-specific CTLs were tested by staining of detectable $CD8^+$ /$pentamer^+$ cells with anti Mcl-1/Bim/CD127 antibodies. The HCV specific CTL proliferation ability after specific *in vitro* challenge was tested in the presence and absence of pancaspase inhibitor z-VAD-fmk. Gamma-interferon production after specific in vitro challenge according to

CD127 expression level was also tested in these cells. All the stained cells were analyzed by flow cytometry.

As per the aim of this study, analysis of the pentameric cells in HCV infected patients was done. For this analysis the minimum sensitivity of the pentameric complexes to detect HCV specific CTLs was carried out. A group of 10 subjects with HLA-$A2^-$ chronic HCV infection was used as negative control. The cut-off was considered as the average frequencies of the HCV-specific $CD8^+$ T cells ($Pent^+$) detected in those cases plus two times standard error of the mean. By this definition, the discrimination point was 10 $CD8^+$/ Pent-$NS3_{1406-1415}$ or Pent-$NS3_{1073-1081}$ positive T cells out of 50,000 $CD8^+$ T cells (0.02%). Therefore, it is considered that a subject had detectable cells with this technique, when the count of $CD8^+$/Pent-$NS3_{1406-1415}$ or Pent-$NS3_{1073-1081}$ had more than 10 $Pent^+$ /$CD8^+$ T cells per 50,000 $CD8^+$ T cells. This technique is extremely specific as indicated in Figure V.1, where the staining with HLA-A2/peptide pentameric complex detecting specific cytotoxic cells against HCV NS3 $_{1406-1415}$ epitope after specific stimulation in a chronic HCV HLA-$A2^+$ patient is shown, while this staining did not detect cells after stimulation with CMVpp65 peptide.

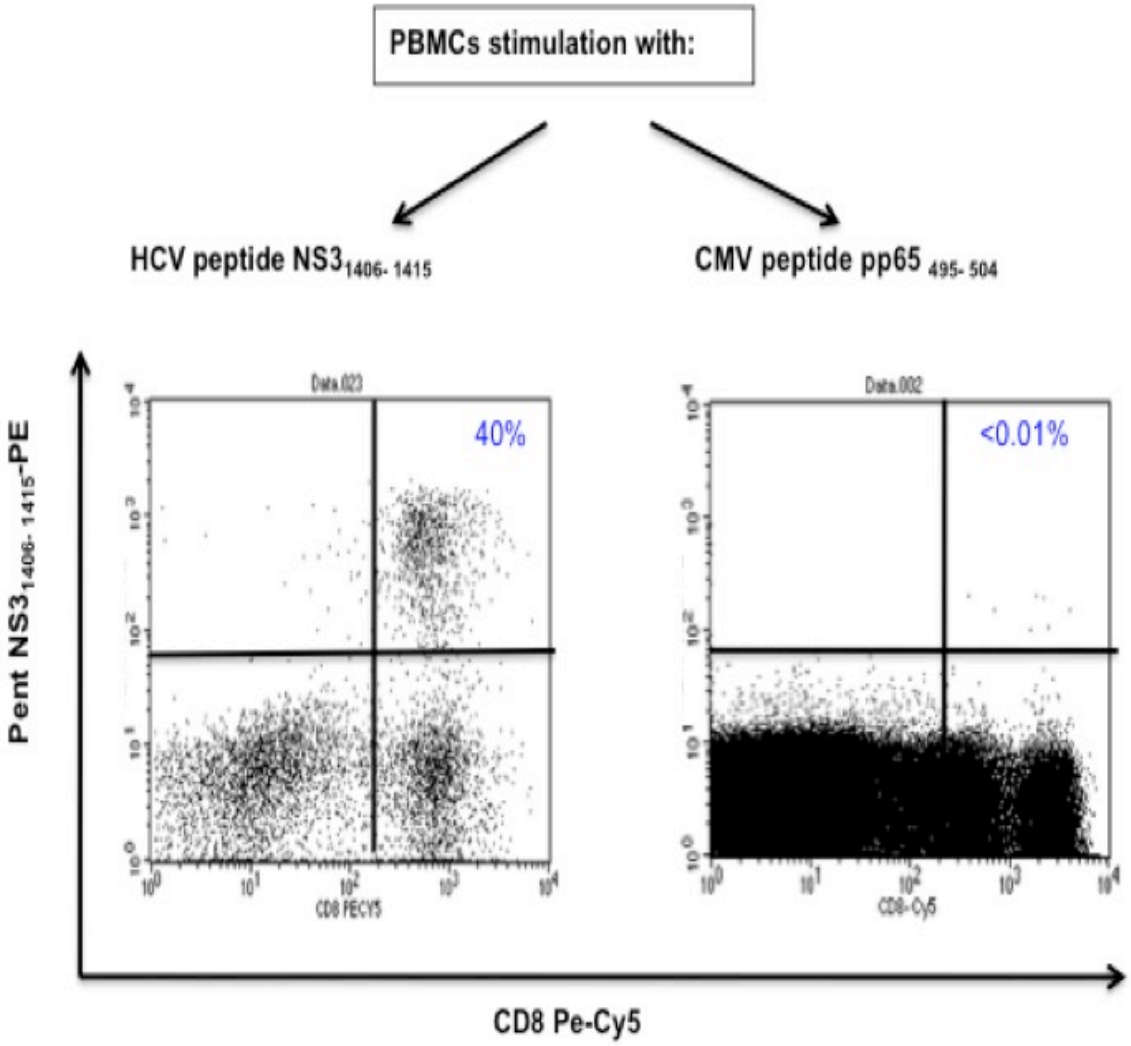


Figure. V.1: Dot plots showing the specificity of $NS3_{1406-1415}$ pentameric complexes to detect $NS3_{1406-1415}$-specific cytotoxic T cells. In PBMC stimulated with $NS3_{1406-1415}$ peptide a double positive cell population $CD8^+$/ pent-$NS3_{1406-1415}^+$ is detected while after stimulating these cells with an HLA-A2 immunodominant CMV epitope no pent-$NS3_{1406-1415}^+$ cells are detected. PBMCs: peripheral blood mononuclear cells. CMV: Cytomegalovirus.

V.1 Study Variables:

V.1.1 Clinical Characteristics of the Groups:

In this study 24 HLA-$A2^+$ HCV^+ genotype 1 patients were included. HCV-specific CTLs against two different HCV immunodominant epitopes were directly *ex vivo* observed in those patients. Thirty-three different $pentamer^+$ populations from these 24 HLA-$A2^+$ HCV genotype-1 patients with directly ex vivo detectable $pentamer^+$ cells were recruited for this study. Of these 33 different HCV- specific CTL populations, 20 of them had CTLs specific against $NS3_{1406–1415}$ and another 13 had CTLs specific against $NS3_{1073–10.}$ To study the functional characteristic of $pent^+/CD8^+$ T cells with respect to CD127 expression level, study's samples were split into two groups according to CD127 expression. On the basis of our previous work, 20 MFI units for CD127-phenotype staining was chosen as cut-off to differentiate high and low expressing CD127 cells. $CD127^{high}$ expression level was considered more than 20 MFI for CD127-expression on $pentamer^+/CD8^+$ cells and CD127 low group was showing less than 20 MFI for CD127 expression on $pentamer^+/CD8^+$ cells. In that work, we showed that during persistent infection a $CD127^{low}$ population was observed in peripheral blood, while $CD127^{high}$ expressing cells were present predominantly in patients controlling infection. In some chronic HCV cases, unexpected $CD127^{high}$ expressing HCV specific CTLs were observed but after HCV-epitope sequencing we realize that this high CD127 expression was due to absence of immunological pressure. In

this study from the 33 HCV-specific CTL populations, 14 cases were CD127^{high} phenotype while 19 cases were CD127^{low}.

Table V.1: Clinical and virological features of the groups of the study.

	CD127 high N=14	CD127 low N=19	P value*
Age (years old)	43 (4)	46 (15)	0.014
Sex (M/F) (%)	78/22	77/23	NS
Diagnosis (%) • SVR • CHC	 86% 14%	 5% 95%	<0.001
Time of evolution (years)	24 (6)	29 (24)	NS
ALT (IU/mL)	40 (31)	72 (82)	<0.001
HCV viral load (IU/mL) (log scale)	1.6 (0.8)	6.06 (0.96)	<0.001
Genotype 1a/1b (%)	30/70	55/45	NS
Epitopes tested ($NS3_{1406}$/$NS3_{1073}$) (%)	73/26	54/46	NS

CHC: chronic hepatitis C, CTL: cytotoxic T lymphocyte, F: female, HCV: hepatitis C virus, M: male, N: number of samples, NS: non-significant, SVR: sustained virologic response, *Mann-Whitney U test.

Both CD127 groups were similar with respect to principle demographic characteristics for example patients' age, disease evolution time and sex

distribution (Table V.1). The average age of the study subjects stood at the end of fourth decade in two groups, ranging from 43 years to 46 years. The percentage of males was higher than female in both groups, namely for $CD127^{high}$ expressing group, 78% were male and 77% in $CD127^{low}$ expressing group. Furthermore, the evolution time of disease was similar in studied groups, ranging from 20 to 30 years. In summary, these data showed that there was consistency in clinical variables between the compared groups. In addition, the selected HLA-$A2^{+}$ HCV^{+} populations were having specifically the same genotype 1a/1b distribution.

V.2 Viral replication and liver damage according to CD127 expression on pentamer^{+} cells:

In the viral infection, CD127 expression, as a survival marker, could decide the fate of viral specific $CD8^{+}$ T cells. In addition, CD127 expression could affect to viral load and effectivity of viral specific T cells on destroying targeted liver cells. CD127 expression level on HCV specific $CD8^{+}$ T cells was analyzed by studying mononuclear cells from the patients' peripheral blood samples. PBMCs were double stained directly *ex-vivo* with anti-CD8-PeCy5 mAbs and PE-pentamers to test the presence of HCV-specific $CD8^{+}$ cells against the two different HCV specific epitopes. The pentameric positive cells were further stained with anti-CD127-FITC mAbs. Depending upon the CD127 expression level, these samples were split into two groups and viral load and ALT level were compared to check viral control and liver damage according to CD127 expression.

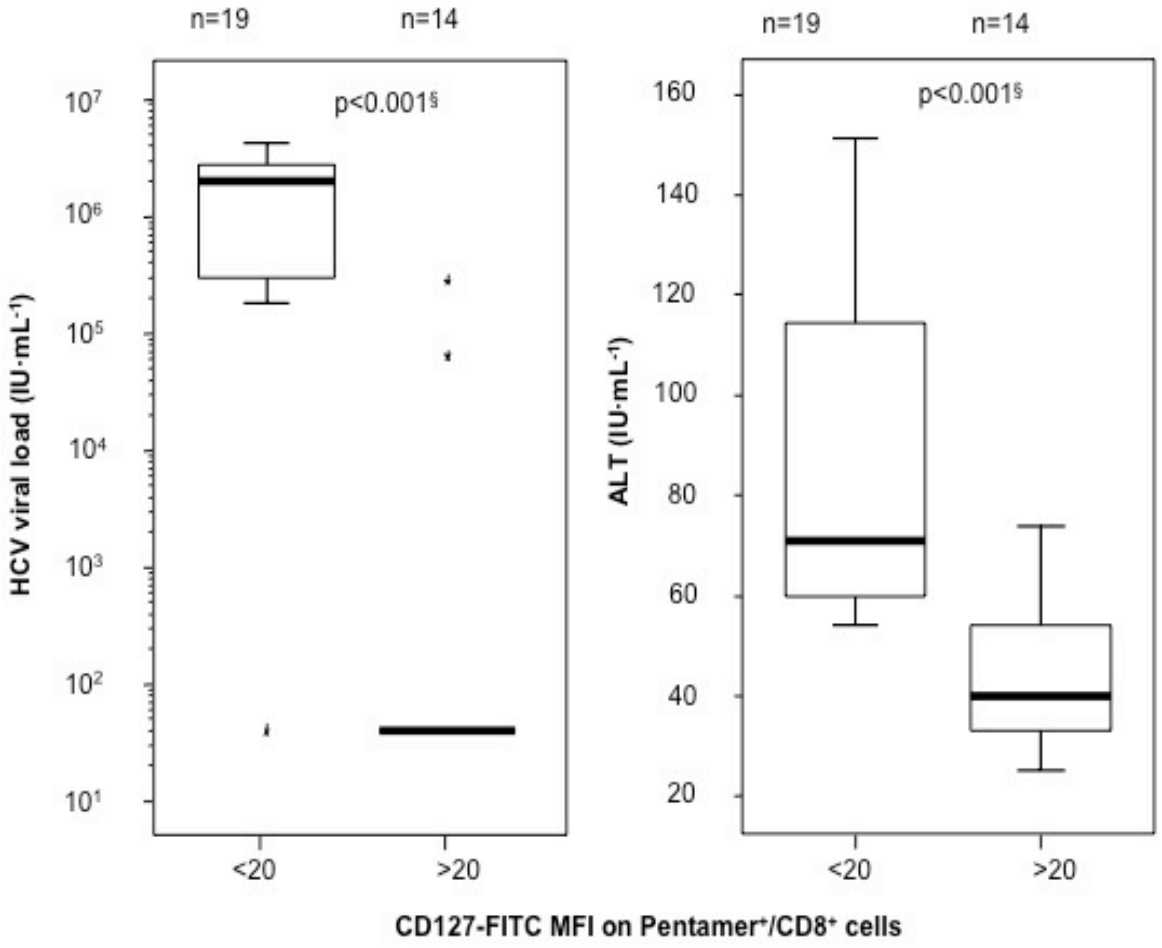


Figure. V.2: Liver damage and viral control according to CD127 expression on HCV-specific cytotoxic T cells: Box plots showing serum ALT (IU/mL) and viral load (IU/mL) levels according to the CD127 expression on HCV-specific cytotoxic T cells (pentamer$^+$/CD8$^+$ cells). §Mann–Whitney U test; MFI, mean fluorescence intensity; n, number of cases.

HCV individuals with high expression of CD127 were having undetectable or low viral load, while HCV specific CTLs from $CD127^{low}$ expressing group associated with high viral load. The average HCV viral load of $CD127^{low}$ group population was more than 10^6 IU/ml, whereas for $CD127^{high}$ group was less than 10^2 IU/ml, which is almost three times lower than in $CD127^{low}$-expressing individual group, which showed significant P value about $<0.001^{\zeta}$. In case of liver damage according to CD127 expression level, $CD127^{high}$ group had low ALT (about 40 IU/ml), while $CD127^{low}$ group was showing high ALT (about 70 IU/ml), with p value less than 0.001 ($P<0.001\zeta$) (Fig. V.2 and Table V.1).

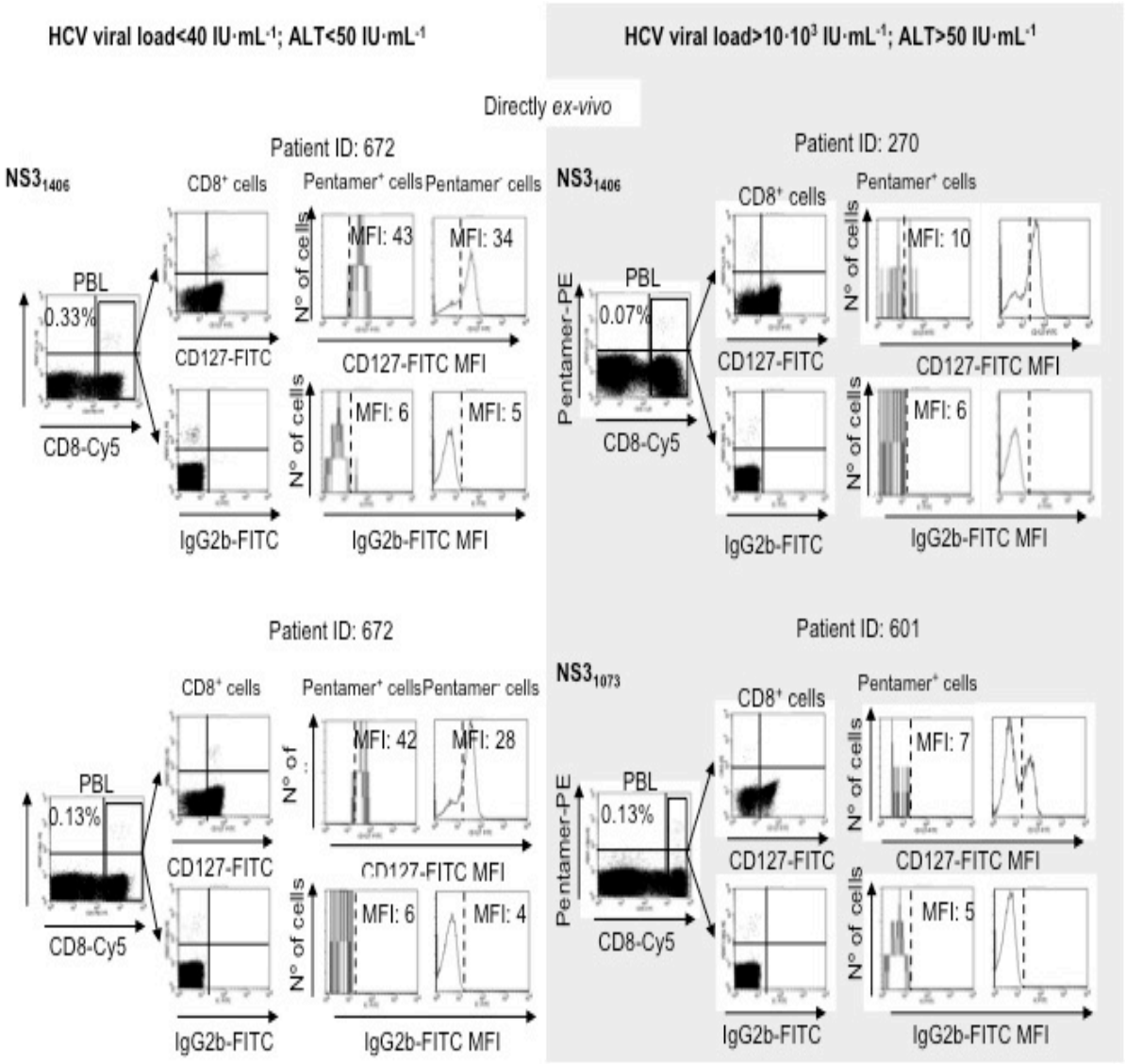


Figure. V.3: Representative FACS dot plots and MFI histograms of peripheral T cells stained with labeled mAb against CD8 and CD127 and with pentameric HLA-A2/peptide PE-labeled complexes against NS31406 and NS31073 HCV epitopes from cases with different degrees of liver damage and viral control. The figure on the upper left corner of the FACS dot plots for pentamer/CD8 staining shows the frequency of pentamer$^+$ cells out of the total CD8$^+$ cells. FACSÒ histograms are gated on pentamer$^+$ and pentamer- CD8$^+$ cells. The dashed line in the histograms represents the upper limit of the staining with the isotypic control. ID, patient identification; MFI, mean fluorescence intensity; n, number of cases; PBL, peripheral blood lymphocytes.

The CD127 expression on pentamer^{+} cells in the CD127^{low} group was almost three times lower (MFI, 13.90; IQR, 8.37) than in the CD127^{high} group (MFI, 34.59; IQR, 10.02) (P < 0.001; Figs V.4 and V.11) and similar to the MFI observed with the isotype control staining (MFI, 5.60; IQR, 4.30) (P > 0.05). (Fig V.3). The pentamer^{+} cells were showing significant difference in CD127 expression level, but the total CD8^{+} cells were showing similar CD127 expression in respective groups, therefore, this difference was HCV antigen specific. (Fig. V.4).

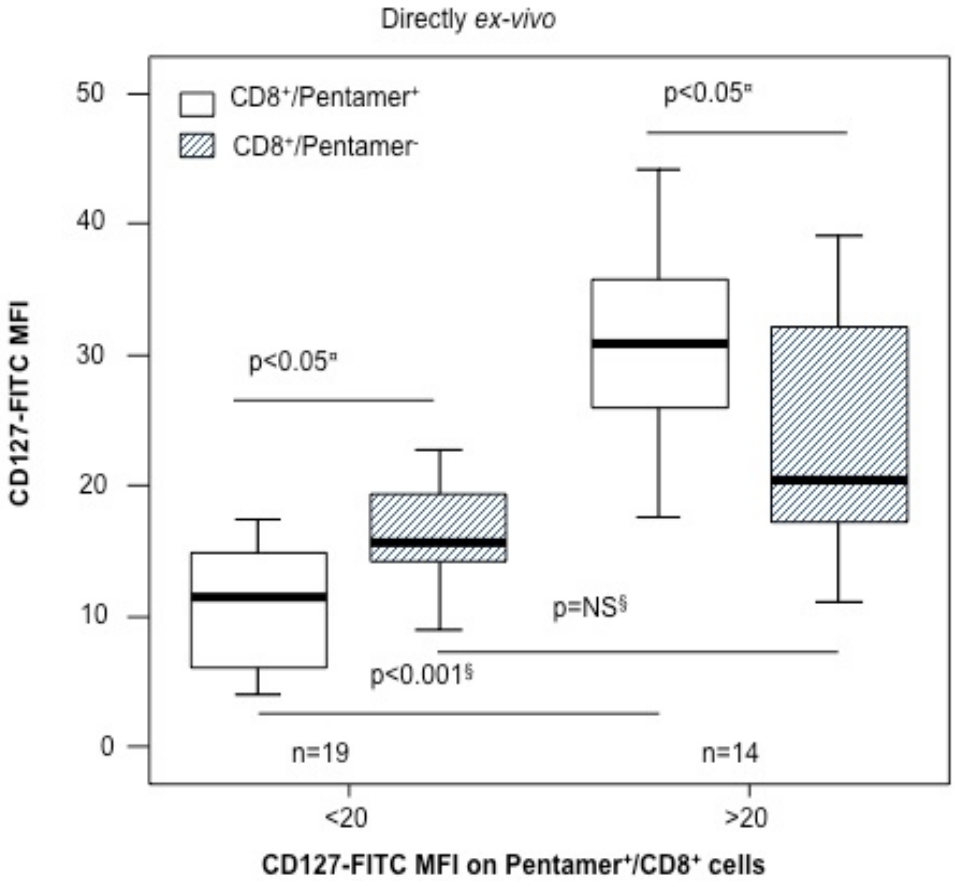


Figure. V.4: Box plots showing the directly ex vivo CD127 MFI on total CD8^{+} and pentamer^{+} cells in the CD127 expression level groups of the study. §Mann–Whitney U test. ¤Wilconxon test. MFI, mean fluorescence intensity; n, number of cases.

Therefore, $CD127^{low}$-expressing HCV-specific CTLs associated with high HCV viraemia, while $CD127^{high}$ expression correlated with undetectable viral loads ($P < 0.001$). Similarly, the CD127 low expression level also associated with high liver damage, whereas high expression of CD127 in HCV-specific CTLs correlated with low serum ALT value. In fact, most populations with $CD127^{high}$-expressing $pentamer^{+}$ cells were sustained virologic responders, tested at least 6 months after finishing standard anti-HCV treatment, while $CD127^{low}$- expressing cells were mainly present in naive chronic hepatitis C infected cases. Thus, the CD127 expression level in HCV specific CTLs is clearly associated with the liver damage and viral replication in HCV patients.

V.3 Reactivity of $pentamer^{+}$ cells according to CD127 expression level:

To test whether CD127 expression level could affect HCV-specific CTL reactivity after antigen encounter because of apoptosis activation, proliferation ability of HCV specific $pentamer^{+}$ cells was analyzed after stimulation with HCV specific *in vitro* challenge with or without apoptosis blockade. Ten cases of each group were analyzed after HCV specific *in vitro* challenge by HCV specific peptide, in presence of either a pancaspase inhibitor (z-VAD-fmk) or β-galactosidase as control. Consequently, frequency of HCV specific CTLs according to the expression of CD127, directly ex vivo and after specific in vitro stimulation were compared to check the proliferation ability of those cells in these conditions.

In directly *ex vivo* and in vitro stimulation, the peripheral frequency of HCV specific CTLs was analyzed and found significant differences. The peripheral frequency of pentamer^{+} cells out of the total CD8^{+} cells was very low directly ex vivo and similar in both groups; CD127^{low}: 0.05% (IQR, 0.09) and CD127^{high}: 0.04% (IQR, 0.05) ($P > 0.05$, Figs V.5 and V.7). However, the frequency of CD127^{high}-expressing pentamer^{+} cells was higher than in CD127 ^{low} expressing pentamer^{+} cells after stimulation with the specific peptides for 10 days without z-VAD-fmk treatment. In CD127^{high} expressing group, the median frequency of pentamer^{+} cells was 1.7% (IQR, 6.77), whereas only 0.003% (IQR, 2.9) was found in CD127^{low} group ($P < 0.05$, Figs V.6 and V.7). Furthermore, only 30% of the low CD127 expressing cases showed a positive proliferation as a cluster of pentamer^{+} cells, while in 80% of samples from CD127 high expressing samples, proliferation was observed ($P < 0.05$, Fig V.9).

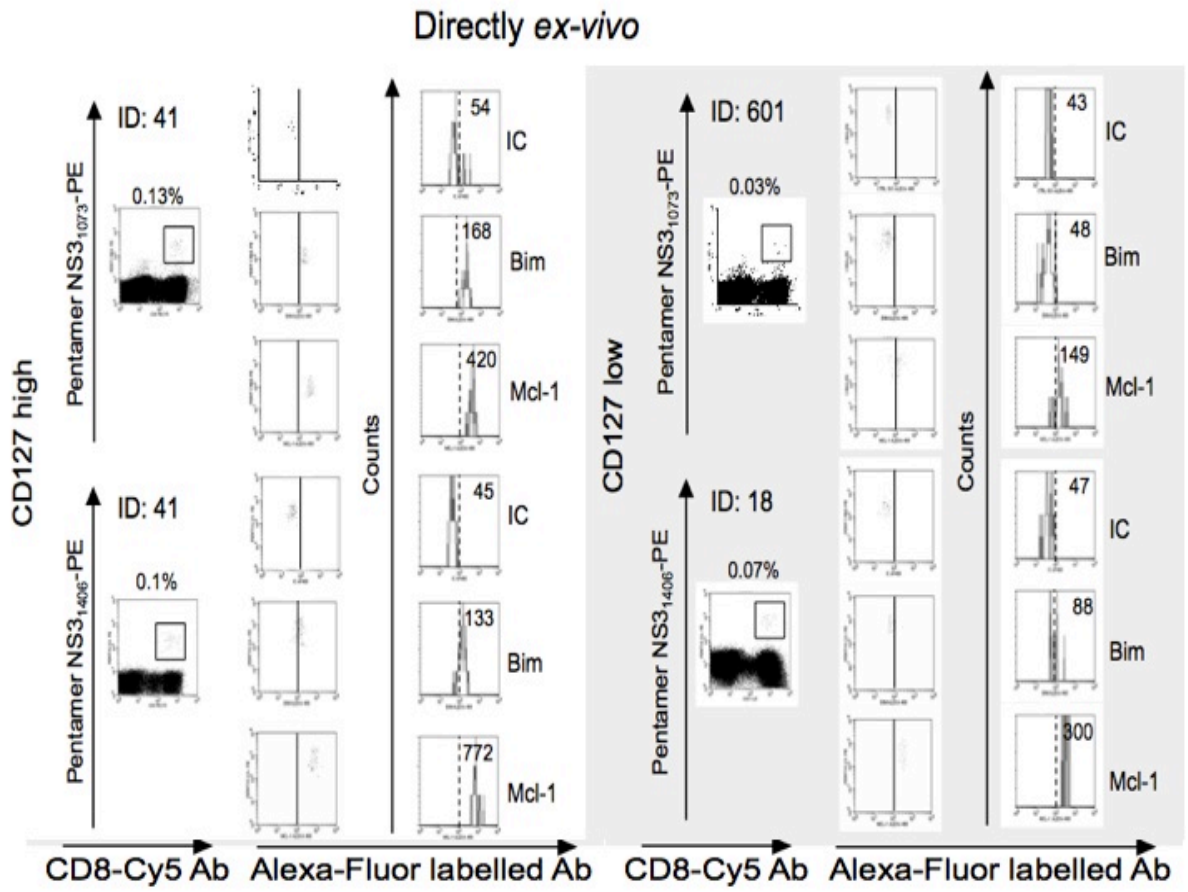


Figure. V.5: Representative FACS dot plots and mean fluorescence intensity (MFI) histograms showing the Mcl-1 and Bim expression on peripheral $CD8^+$/$pentamer^+$ cells, directly ex vivo with regard to the CD127 expression level on those cells. The Figure on the top of the dot plots represents the frequency of $pentamer^+$ cells out of the total $CD8^+$ cells. The Figure in the upper right corner in the histogram plot represents the MFI for Bim and Mcl-1 staining. The continuous and dashed line in the dot plots and histograms represent the cut-off point for positive staining according to the negative control. IC, isotypic control; ID, patient identification.

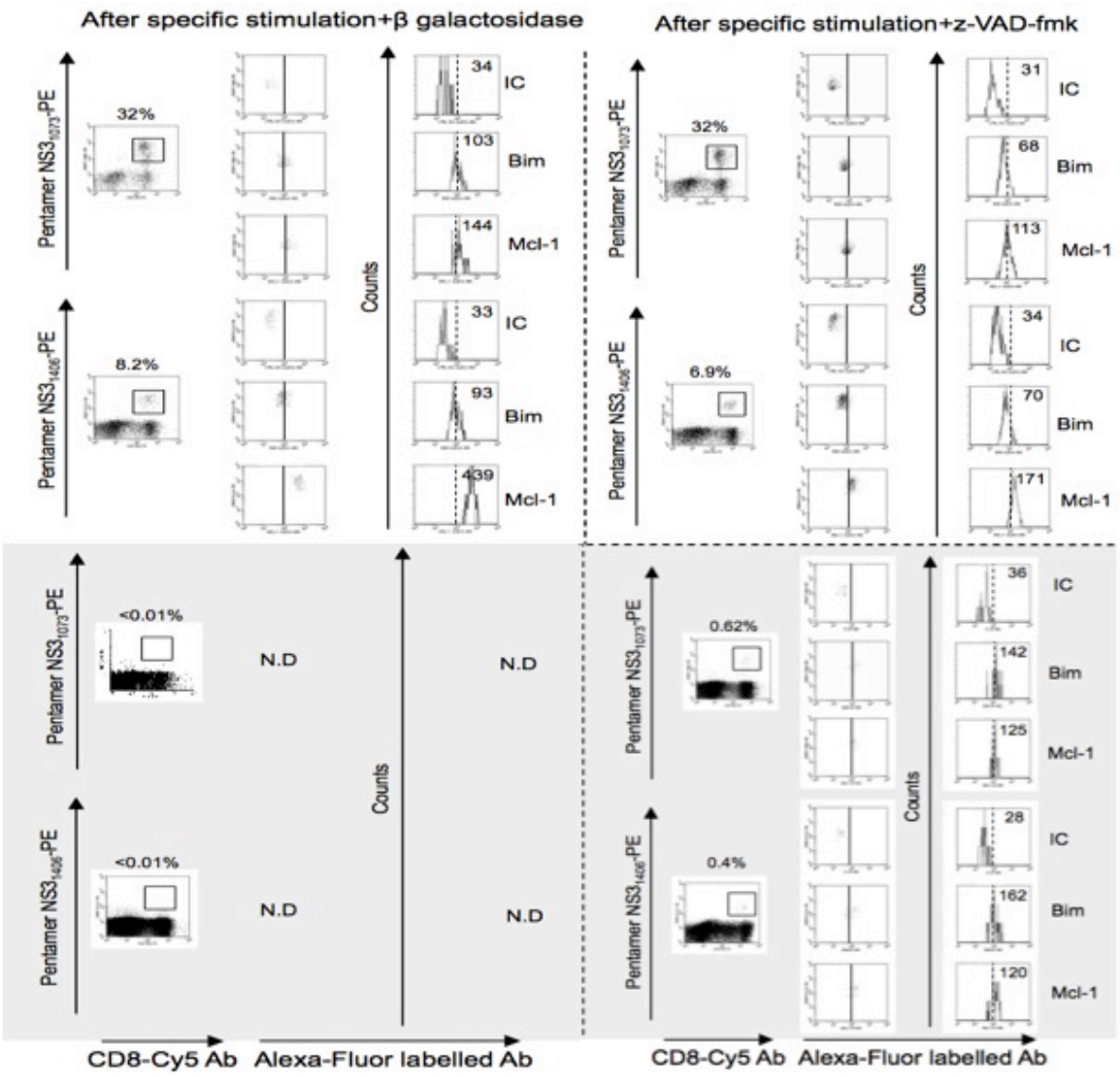


Figure. V.6: Representative FACS dot plots and mean fluorescence intensity (MFI) histograms showing the Mcl-1 and Bim expression on peripheral $CD8^+$/$pentamer^+$ cells, after specific in vitro challenge, with regard to the CD127 expression level on those cells. Peripheral blood lymphocytes were subjected to specific in vitro challenge in the presence of z-VAD-fmk and β-galactosidase as control. The Figure on the top of the dot plots represents the frequency of $pentamer^+$ cells out of the total $CD8^+$ cells. The Figure in the upper right corner in the histogram plot represents the MFI for Bim and Mcl-1 staining. The continuous and dashed line in the dot plots and histograms represent the cut-off point for positive staining according to the negative control. IC, isotypic control; ID, patient identification; N.D., not done because of lack of $pentamer^+$/$CD8^+$ proliferation after specific stimulation.

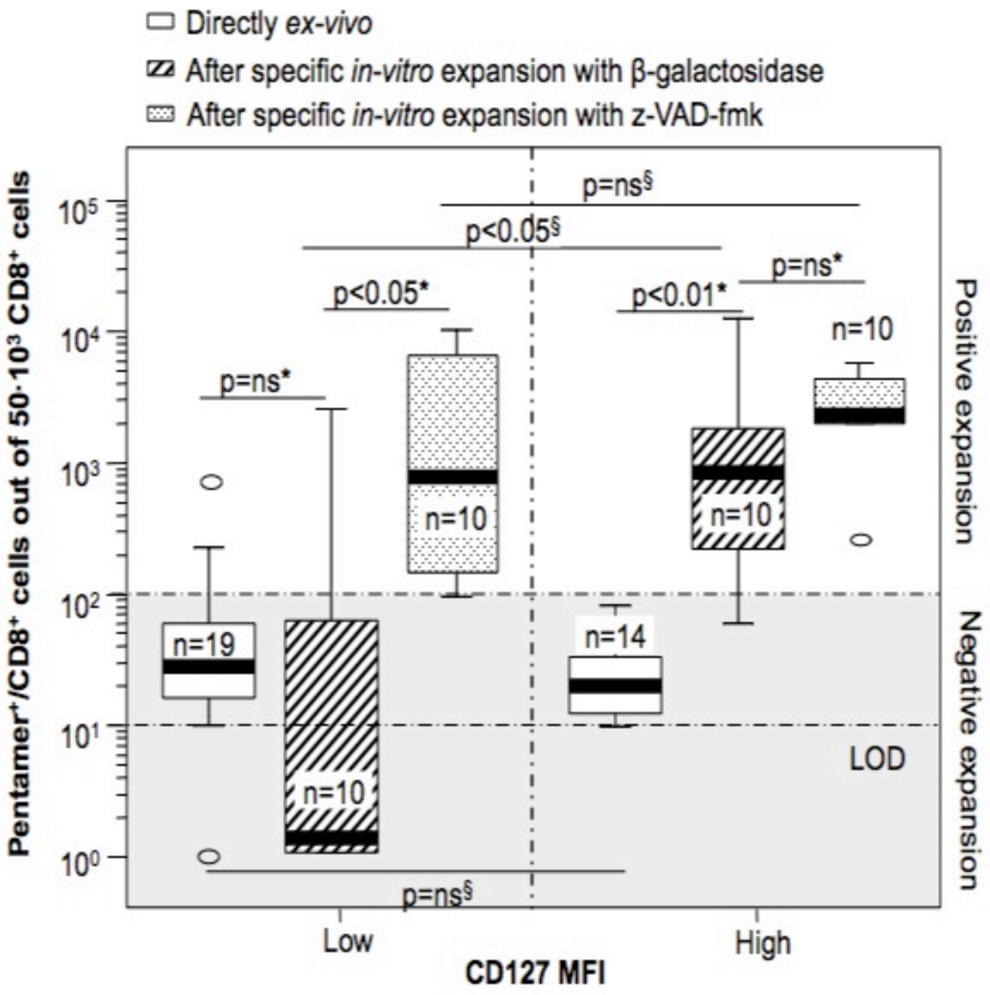


Figure. V.7: Reactivity of $CD8^+$/$pentamer^+$ cells according to CD127 expression level: Box plots showing the frequency of $CD8^+$/ $pentamer^+$ cells directly ex vivo and after specific in vitro challenge in the presence of either z-VAD-fmk or β-galactosidase as control in high and low CD127-expressing $pentamer^+$ cells. *Wilconxon test. §Mann–Whitney U test. LOD, limit of detection; MFI, mean fluorescence intensity; n, number of cases; n.s., non-significant; O, outliers.

On the other hand, the pancaspase inhibitor works for inhibiting apoptosis and improved the proliferation ability after specific stimulation. In presence of pancaspase inhibitor –z-VAD-fmk in proliferation set up, $CD127^{low}$-expressing $pentamer^{+}$ cells improved in expansion ability after specific stimulation (1.39%; IQR, 13.2), with respect to the observed expansion without this pancaspase inhibitor ($P < 0.05$, Figs V.6 and V.9). The observed expansion with pancaspase inhibitor in $CD127^{low}$ expressing samples, reached a similar expansion level to the $CD127^{high}$ expressing $pentamer^{+}$ cells (4.5%; IQR, 6.25) in absence of pancaspase inhibitor –z-VAD-fmk ($P > 0.05$, Figs V.6 and V.9). Moreover, the percentage of experiments with $pentamer^{+}$ cell proliferation augmented after z-VAD-fmk treatment up to 80% ($P < 0.05$) (Figs V.6 and V.9), in the $CD127^{low}$ group, showing a similar expansion frequency to the one observed in the $CD127^{high}$ group ($P > 0.05$) (Fig. V.9). Treatment of z-VAD-fmk did not only affect on $CD127^{low}$ group but also on $CD127^{high}$ group. The z-VAD-fmk treatment also non-significantly enhanced proliferation of $CD127^{high}$ expressing cells ($P > 0.05$, Figs V.6 and V.9). Interestingly, CD127 expression has found to decrease in $CD127^{high}$ expressing $pentamer^{+}$ cells ($P < 0.001$) and reached to a similar level (MFI, 8.05; IQR, 5.90) to the one observed directly *ex vivo* in the CD127 low group, after specific in vitro stimulation (Figs V.10 and V.11).

Therefore, proliferation of $CD127^{low}$ $pentamer^{+}$ cell after specific *in vitro* challenge was impaired ($P < 0.05$), although this was corrected by z-VAD-fmk treatment ($P < 0.05$). Thus, the reactivity of HCV specific $pentameric^{+}$ cells correlated with CD127 expression level.

V.4. Interferon-γ production by pentamer^{+} cells according to CD127 phenotype:

The level of Interferon-γ (IFN- γ) production significantly correlates with the level of viral-specific CTL activity. The effectivity of viral specific cells is measured by the percentage of IFN- γ producing HCV specific CTLs. To explain, whether the CD127 expression level could affect on effectivity of HCV-specific cells, the IFN-γ production on CD127 expressing CTLs was carried out. Directly *ex vivo* IFN- γ producing pentamer^{+} cells were analyzed in 11 cases (5 cases in CD127^{high} and 6 cases in CD127^{low} group). The frequency of IFN-γ producing pentamer^{+} cells was directly proportional to the CD127 expression in pentamer^{+} cells. CD127^{low} expressing pentamer^{+} cells were showing lower frequency of IFN-γ producing pentamer^{+} cells (40%; IQR, 32) than in the CD127^{high} group (61%; IQR, 13.5) ($P < 0.05$, Fig. V.8).

Therefore, the frequency of IFN- γ producing pentamer^{+} cells was lower in the CD127^{low} than in the CD127^{high} group. Thus, the correlation of percentage of frequency of IFN- γ production in HCV specific cells is directly proportional to the CD127 expression level.

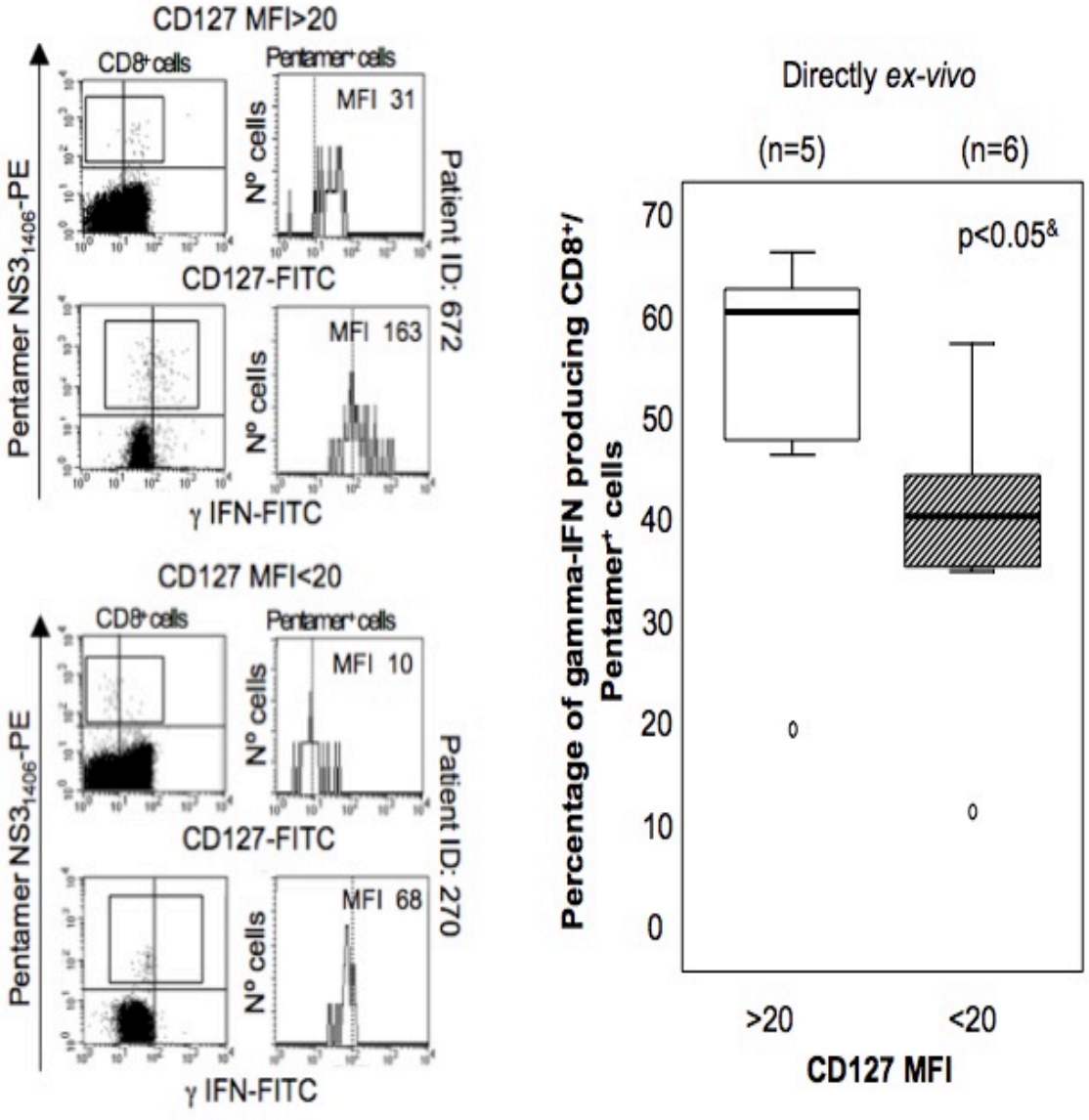


Figure. V.8 (A) Representative FACS dot plots and mean fluorescence intensity (MFI) histograms showing directly ex vivo interferon-γ (IFN-γ) production on pentamer$^+$ cells according to CD127 expression. (B) Box plots showing the percentage of IFN-producing pentamer$^+$ cells, in relation to CD127 expression level. The Figure in the upper right corner in the histogram plot represents the MFI for IFN γ staining. §Mann–Whitney U test; MFI, mean fluorescence intensity; ID, patient identification; n, number of cases.

V.5. Mcl-1 and Bim expression according to CD127 phenotype:

The observation of reactivity impairment on pentamer$^+$ cells according to low CD127 level could be related to apoptosis induction. In intrinsic apoptosis pathway, the known Bcl-2 family proteins, Mcl-1 and Bim, are shown to be involved. To explain the different T cell reactivity according to CD127 level, anti-apoptotic molecule- Mcl-1 and pro-apoptotic molecule- Bim phenotypes were analyzed on pentamer$^+$ cells. Bim and Mcl-1 were stained with Alexa-Flour 488 labeled antibody. Bim and Mcl-1 MFI on gated CD8$^+$/Pentamer$^+$ cells were calculated. A value, which is higher than the MFI mean plus 2 standard deviations obtained from the pool of pentamer$^+$ cell staining with the isotypic Antibody control (Cut-off level: 90 unit of fluorescence) was considered positive for those phenotypes.

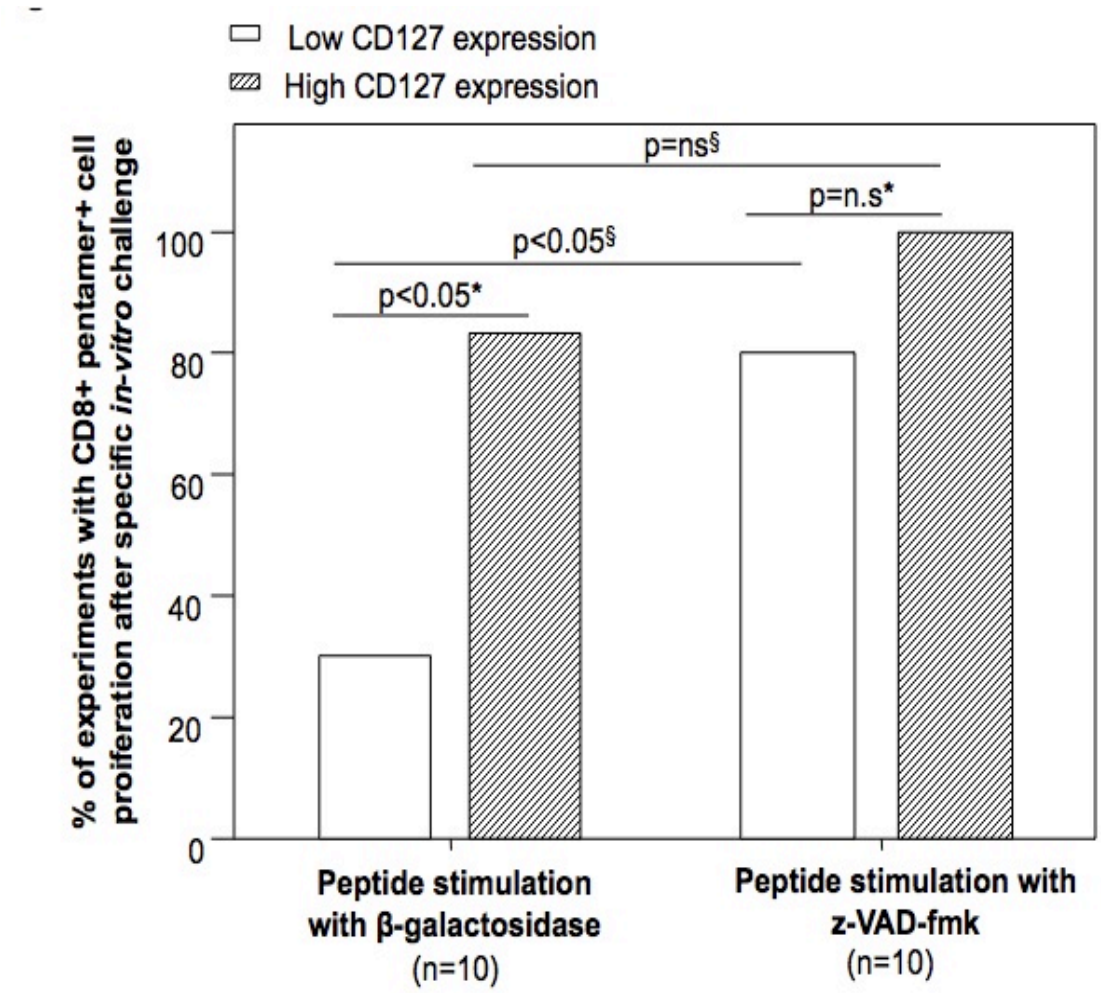


Figure. V.9: Bar plots showing the percentage of experiments with $CD8^+$/$pentamer^+$ cell proliferation after specific in vitro challenge in the presence of either z-VAD-fmk or β-galactosidase as control, with regard to CD127 expression level. Positive proliferation was considered when more than 0.2% of $CD8^+$/$pentamer^+$ cells in a clear cluster were observed after specific stimulation. *Wilconxon test. §Mann–Whitney U test. n, number of cases; n.s., non-significant.

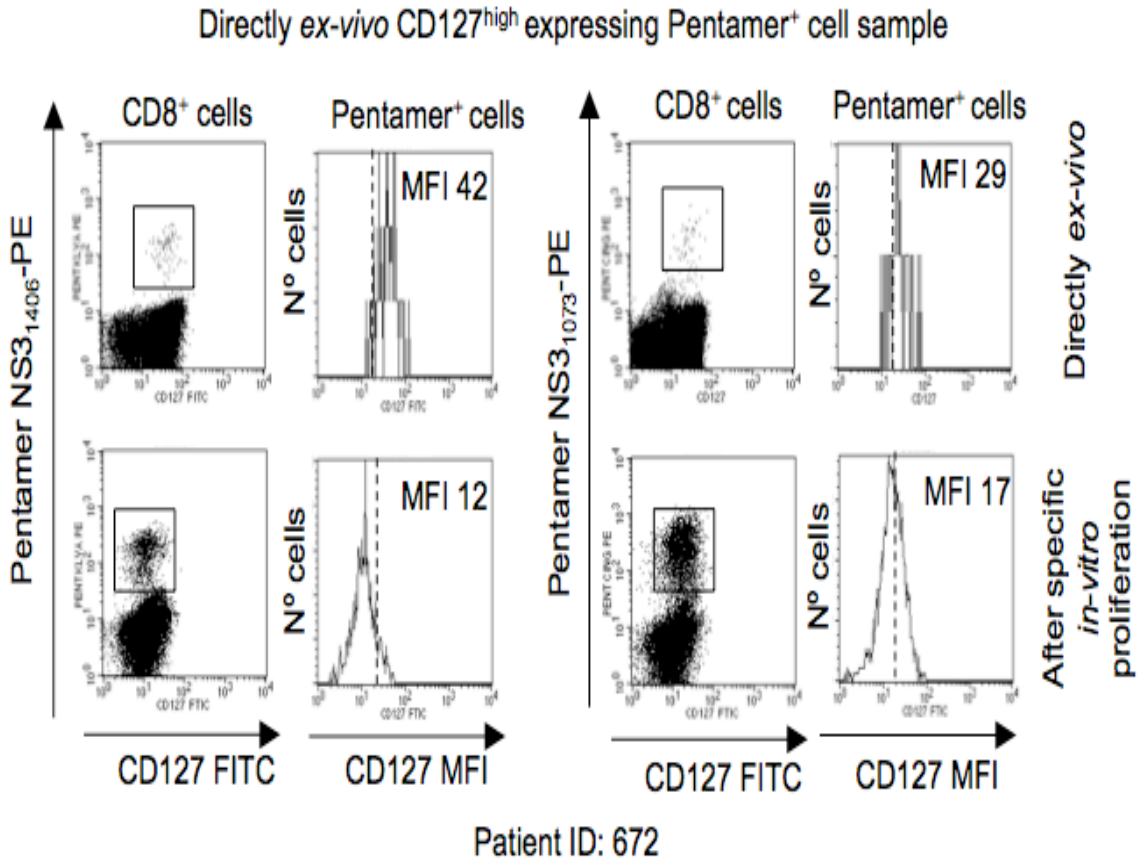


Figure. V.10: Representative FACS dot plots and MFI histograms from CD127high-expressing pentamer^{+} cells, with CD127 expression on CD8^{+}/pentamer^{+} cells directly *ex vivo* and after specific in vitro challenge. MFI, mean fluorescence intensity; ID, patient identification.

The comparison of directly *ex vivo* Bim and Mcl-1 expression on peripheral pentamer^{+} cells according to CD127 level was analyzed. Directly *ex vivo*, Bim expression was similar on peripheral pentamer^{+} cells in both CD127 expressing pentamer^{+} cell groups. Particularly, Bim expression on pentamer^{+} cells was showing MFI about 79.19 (IQR, 117.60) in CD127^{low}-expressing cells and about 156.20 MFI (IQR, 136.01) was in case of CD127^{high} expressing group ($P > 0.05$, Figs V.5 and V.11).

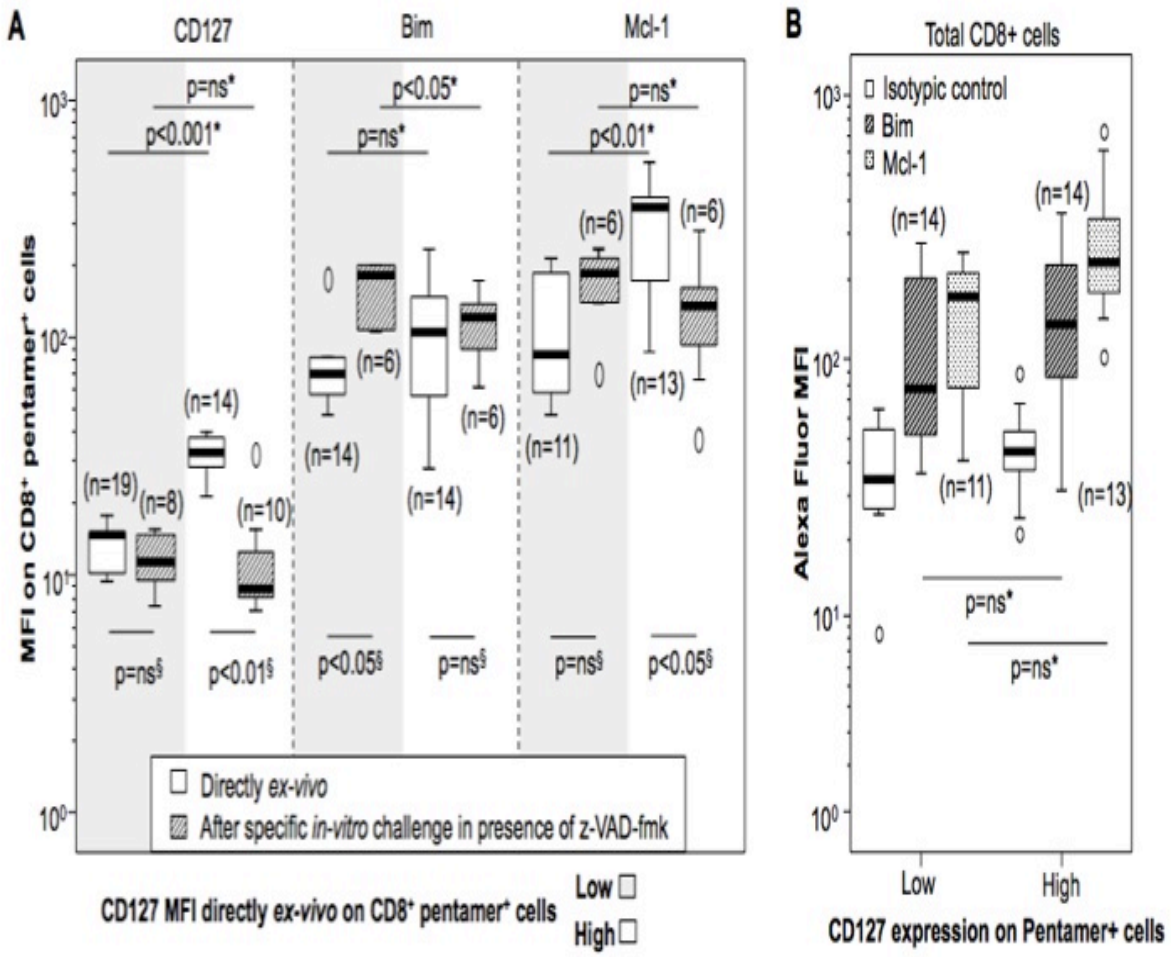


Figure. V.11: Mcl-1 and Bim expression on $CD8^+$/$pentamer^+$ cells according to CD127 expression level and their relationship with $CD8^+$/$pentamer^+$ cell reactivity after antigen encounter: A) Box plots showing the MFI for CD127, Mcl-1 and Bim staining on peripheral $CD8^+$/$pentamer^+$ cells directly ex vivo and after specific in vitro challenge, blocking apoptosis, according to CD127 expression level. (B) Box plots describing the directly ex vivo Mcl-1/Bim expression on total $CD8^+$ cells in the two groups of the study. §Wilconxon test. *Mann–Whitney U test; MFI, mean fluorescence intensity; n, number of cases; n.s., nonsignificant.

On the other hand, in case of Mcl-1, directly *ex vivo* Mcl-1 expression level was different between groups according to CD127 expression level. The Mcl-1 expression was directly proportional to the CD127 expression level. Directly *ex vivo,* Mcl-1 expression was significantly lower on $CD127^{low}$ $pentamer^{+}$ (MFI, 172.80; IQR, 119.57) than on $CD127^{high}$-expressing cells (MFI, 278.92; IQR, 266.65) ($P < 0.01$, Figs V.5 and V.11).

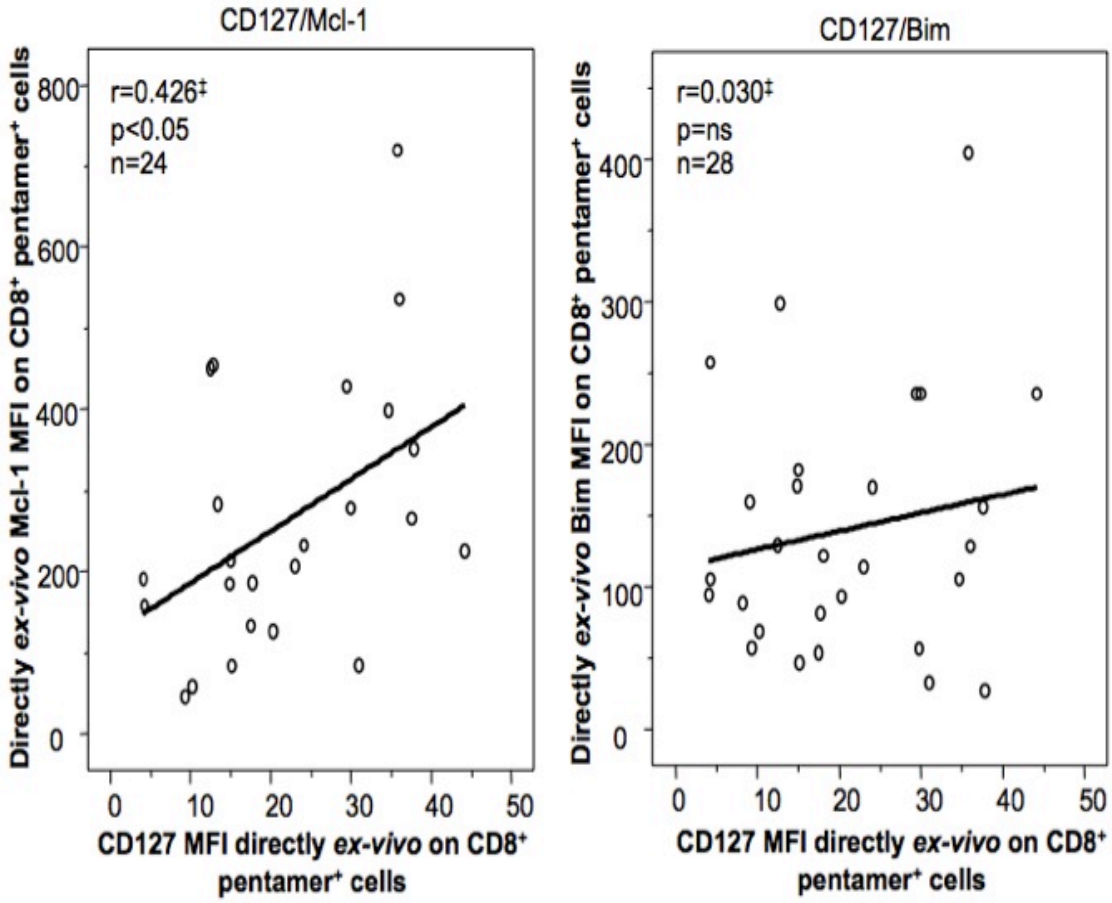


Figure. V.12: Scatter plots showing the correlation between CD127 expression on $CD8^{+}$/$pentamer^{+}$ cells and Mcl-1/ Bim expression on peripheral $CD8^{+}$/$pentamer^{+}$ cells. ‡ Spearman's correlation coefficient; MFI, mean fluorescence intensity; n, number of cases

Thereafter, the correlation between CD127 expression level and Mcl-1 or Bim expression level on peripheral pentamer$^+$ cells was analyzed. A positive significant correlation between CD127 expression level and Mcl-1 level on peripheral pentamer$^+$ cells ($r = 0.426$, $P < 0.05$) was observed, while no significant correlation between CD127 and Bim expression was observed ($P > 0.05$) (Figs V.12). Simultaneously, the Mcl-1 or Bim expression on total CD8$^+$ cells was also analyzed. CD127 expression level was not inducing any difference in Mcl-1/ Bim expression on total CD8$^+$ cells. In both study groups a similar expression for Mcl-1 or Bim on total CD8$^+$ cells was observed directly *ex vivo*, which gave clear idea that Mcl-1 and Bim expression depending upon CD127 expression level was antigen specific (Figs V.11).

Anti-apoptotic molecule counteracts pro-apoptotic molecule and control apoptotic process. In addition, it is known that Mcl-1 expression could counteract Bim action. Therefore, bearing it in mind, the excess of Mcl-1 expression over Bim level on pentamer$^+$ cells was calculated by subtracting Mcl-1 Alexa-Fluor 488 MFI level to the MFI observed for Bim expression. This calculated variable showed a high significant positive correlation with CD127 expression on pentamer$^+$ cells ($r = 0.648$, $P < 0.001$, Fig. V.13). In fact, the difference between Mcl-1 and Bim expression was almost four times higher on CD127^{high}-expressing pentamer$^+$ cells (difference, 110.74; IQR, 267.57) than on CD127^{low}-expressing pentamer$^+$ cells (32.50; IQR, 99.54) ($P < 0.01$).

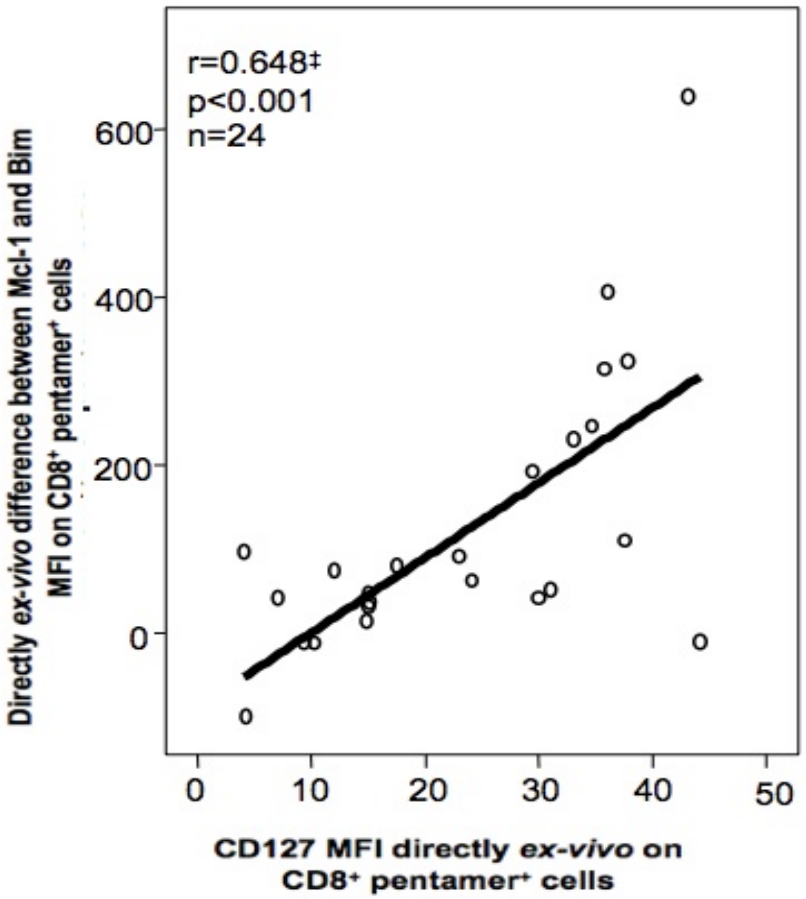


Figure.V.13: Scatter plot showing the positive correlation between the Mcl-1/Bim expression subtraction and CD127 expression level on peripheral $CD8^+$/$pentamer^+$ cells. ‡ Spearman's correlation coefficient. MFI, mean fluorescence intensity; n, number of cases.

According to proliferation ability, in presence of HCV-specific antigen, the excess of Mcl-1 expression regarding to Bim expression level, was analyzed in $pentamer^+$ cells. The excess of Mcl-1 expression, regarding to Bim level, was higher in $pentamer^+$ cells with proliferation ability after antigen encounter (difference, 239.57; IQR, 308.68) than in the cells without reactivity (difference, 52.71; IQR, 102.60) ($P < 0.05$, Figs V.14). Even this profile was

also showing a similar profile to the CD127 level, as far as expansion capacity was concerned. Thus, pentamer^{+} cells with proliferation ability expressed a higher CD127 level (MFI, 29.36; IQR, 22.85) and a higher Mcl-1/Bim expression difference than pentamer^{+} cells without expansion potential, characterized by a low CD127 expression (MFI, 16.80; IQR, 22.85) ($P < 0.05$, Figs V.14).

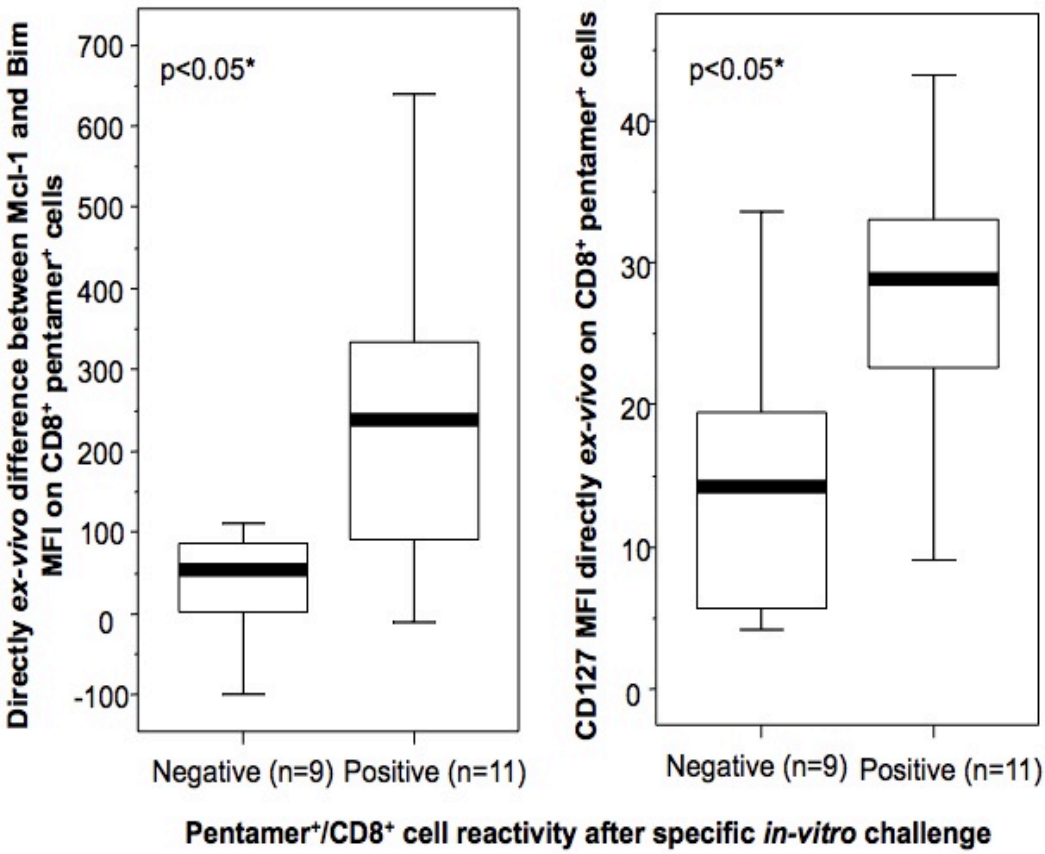


Figure. V.14: Box plots describing the CD127 expression level and Mcl-1/Bim expression difference on peripheral CD8^{+}/pentamer^{+} cells with regard to their reactivity after antigen encounter. * Mann–Whitney U test; MFI, mean fluorescence intensity; n, number of cases.

In presence of z-VAD-fmk and stimulating with HCV specific peptide, whether T cell receptor (TCR)/HLA-I- peptide engagement could influence Mcl-1/Bim imbalance was analyzed, according to CD127 expression level. Therefore, the Mcl-1/Bim/CD127 phenotype, after specific in vitro challenge in the presence of z-VAD-fmk, was also analyzed and compared between both CD127 expression groups. To study Bim and Mcl-1 expression after antigen encounter on $CD127^{low}$-expressing $pentamer^{+}$ cells, z-VAD-fmk was used to ensure the proliferation of these cells. After specific antigen stimulation, in $CD127^{high}$-expressing $pentamer^{+}$ cells CD127 expression was down-regulated ($P < 0.01$) to a similar level to the one observed on $CD127^{low}$ cell, directly *ex vivo*, in association with Mcl-1 down-regulation (MFI, 134.57; IQR, 106.04) ($P < 0.05$), which reached the level described on $CD127^{low}$ cells. However, in case on Bim expression, it remained unchanged (MFI, 121.04; IQR, 79.04) ($P > 0.05$) (Figs V.6 and V.11). On the other hand, in $CD127^{low}$-expressing $pentamer^{+}$ cells, CD127 expression was maintained at low levels after specific in vitro challenge (MFI, 11.28; IQR, 8.26) ($P > 0.05$), and Mcl-1 expression was not modified (MFI, 199.23; IQR, 177.95), but interestingly, Bim expression was significantly up-regulated (MFI, 191.08; IQR, 147.87) ($P < 0.05$) to a significantly higher level than the one expressed by $CD127^{high}$-expressing $pentamer^{+}$ cells ($P < 0.05$) (Fig V.14). These data suggest that Bim expression can be up regulated only after TCR triggering in cells with low CD127 expression.

Therefore, the difference between Mcl-1 and Bim expression on $pentamer^{+}$ cells directly *ex vivo* correlated positively with CD127 expression

level and with pentamer^{+} cell reactivity. To sum up, a low *ex vivo* Mcl-1 expression and Bim up-regulation after antigen encounter are involved in CD127^{low} HCV-specific CTL hyporeactivity during chronic infection, but it can be overcome by apoptosis blockade.

VI

DISCUSSION

VI. Discussion

In HCV persistent infection, specific cytotoxic response is weak and unable to clear the virus [50]. Hepatitis C virus-specific CTLs are essential effector tools to control HCV infection, but the lack of a broad and strong $CD8^+$ T-cell response has been linked to persistence of HCV infection [108, 123]. In addition, sustained immunological viral pressure in chronic non-cytopathic viral infection induces an anergic phase and a subsequent deletion on specific CTL [242], i.e. prone to apoptosis during persistent infection [121, 243]. This phenomenon may be related to a high, long-term activation of these cells during persistently elevated viraemia, conducting to a exhausted $CD8^+$ T cell response during chronic HCV infection [168, 244]. In fact, upon continued antigenic stimulation, $CD8^+$ T cells lose their effector function and are selectively deleted, resulting in failure to achieve viral clearance, and this fact correlates with CD127 down-regulation and resulted in deletion of antigen specific naïve and memory T cells [245]. This could be a physiological strategy by the host to avoid immune-mediated injury during chronic infection. Therefore, in persistent HCV infection, peripheral HCV-specific CTLs could acquire an exhausted phenotype to avoid a harmful immune response. Subsequently, these cells cannot withstand further activation in the liver after antigen re-encounter, leading to selective T cell deletion.

The procedure of T cell death during chronic viral infection is determined by a carefully balanced and complex group of pro- and anti-apoptotic proteins of the Bcl-2 family, such as Bim and Mcl-1 [234]. Bim is clearly involved in intrahepatic-specific CTL apoptosis in animal models [172]. Bim is thought to

act principally by activating Bax, permitting mitochondrial release of cytochrome c, which activates caspases, ultimately leading to cell death [234]. Expression of Bim is up-regulated in human T cells in response to TCR triggering by the protein kinase C (PKC) and calcineurin pathways [199]. Nevertheless, there are other mechanisms involved in Bim up-regulation during chronic infection, such as the effect of certain cytokines. IL-7 withdrawal could induce Bim-mediated apoptosis. In fact, in a persistent viral infection animal model, Bim-mediated apoptosis correlates with low IL-7 receptor expression on specific T cells [200]. It has been proved that Bim is required for switching off $CD8^+$ T cell response in the setting of a superantigenic stimulus [246] or an acute viral infection [247] in murine model. Furthermore, Bim has also recently been found to regulate $CD8^+$ T cell responses during chronic LCMV infection in mice [213], which is more relevant to the situation of chronic HCV infection. Bim mediates apoptosis of $CD127^{low}$ effector T cells [200], which is the main responsible of $CD127^{low}$ expressing virus specfic $CD8^+$ T cells apoptosis [248]. Transforming growth factor (TGF)-β is also involved in Bim-mediated apoptosis [219]. Therefore, all these data indicate that Bim action is not only modulated through TCR stimulation, but also by other regulatory mechanisms such as either IL-7 withdrawal or TGF-β induction. Interestingly, persistent HCV infection is characterized by continuous TCR triggering and CD127 down-regulation on HCV-specific CTLs [235], which could favour, so far, Bim up-regulation. In our work, Bim was up-regulated on $CD127^{low}$-expressing HCV-specific CTLs but not on $CD127^{high}$ cells after antigen encounter, suggesting that TCR triggering can only lead to Bim up-regulation in the absence of IL-7 stimulation on HCV-

specific CTLs. Nevertheless, Bim level is not enough to lead to T cell apoptosis. T cell death also depends on the anti-apoptotic protein expression level, regulated by cytokines, such as IL-7. Regarding this, the Bim pro-apoptotic effect is blocked by the action of the Bcl-2 family of anti-apoptotic proteins such as Mcl-1 and Bcl-2 [214, 237].

CD127 plays an essential role in mature lymphocyte survival by counteracting the induction of apoptosis after antigen encounter through regulation of some of the Bcl-2 proteins, enhancing IL-2 secretion and life span [236]. Therefore, HCV could modulate CD127 expression on HCV-specific CTLs to impair the quality of the adaptive immune response by IL-7 deprivation, as a survival strategy [166, 249]. Mcl-1, induced by IL-7, has been shown to counteract the pro-apoptotic effects of Bim to play an essential role in mature lymphocyte survival [220]. Interestingly, in an experimental model, IL-7 deprivation during stressing conditions leads to Mcl-1 down-regulation on T cells, leading to T cell death that could be avoided by IL-7 treatment [230]. In agreement with this experimental model, our *ex vivo* data show that during persistent HCV infection the predominant peripheral population of HCV-specific CTLs displays a $CD127^{low}$/$Mcl\text{-}1^{low}$ phenotype that remains low after antigen encounter. All in all, these Mcl-1/Bim data could mean that during chronic HCV infection, experienced $CD127^{low}$/ $Mcl\text{-}1^{low}$-expressing T cells attracted to the liver would up-regulate Bim expression after antigen encounter because of the absence of IL-7 stimulus, and subsequently Bim would be released freely to activate Bax, because of the low level of anti-apoptotic proteins such as Mcl-1. Consequently, CD127 level

would play a central role in HCV-specific $CD8^+$ T cell apoptosis by regulation of Mcl-1 expression in vivo and by Bim modulation after antigen encounter. This theory could explain why $CD127^{low}$ HCV-specific $CD8^+$ T cell reactivity from chronic hepatitis C patients is impaired, and theoretically this could be overcome by apoptosis blockade. To test this hypothesis, the correlation between CD127 phenotype, T cell reactivity and Bim/Mcl-1 phenotype on HCV-specific CTLs after apoptosis blockade was tested in this study.

In this work, directly *ex vivo* HCV-specific CTLs from a cohort of patients with different degrees of viral control were obtained. In patients with either low or undetectable viral load, a population of peripheral $CD127^{high}$-expressing cells able to proliferate and to produce IFN-γ after antigen encounter was observed. Theoretically, this population could control HCV traces or be ready for a rapid response in the case of re-infection [250, 251]. These cells were characterized by a higher Mcl-1 expression than the observed Bim level, which could be responsible for maintaining T cell reactivity, avoiding apoptosis activation after antigen encounter. Interestingly, these CD127-expressing cells from resolved patients down-regulated CD127 expression after specific *in vitro* challenge, linked to Mcl-1 down-regulation, without up-regulation of Bim expression, acquiring an *in vitro* phenotype after expansion similar to the one observed in directly *ex vivo* cells from chronic patients. Therefore, these cells could be prone to apoptosis after re-stimulation, mimicking the situation found in persistent infection. On the other hand, in cases with persistent high viral load, HCV-specific CTLs showed a $CD127^{low}$ phenotype that correlated with proliferation and IFN-γ production impairment. This phenotype linked with a

directly *ex vivo* low Mcl-1 expression, similar to Bim levels that could allow apoptosis activation after Bim up-regulation. Actually, after specific *in vitro* TCR triggering, Bim up-regulation on $CD127^{low}$-expressing HCV- specific CTLs was observed. This finding was previously shown by our group [174] and it did not occur on $CD127^{high}$- expressing cells, as previously commented. In an experimental model, a similar observation was made showing that Bim was a critical mediator of apoptosis, induced by TCR re-stimulation, in the context of growth cytokine withdrawal [252]. Therefore, we could speculate that CD127 ^{low} expression on HCV-specific CTLs during persistent infection could favor Mcl-1 down-regulation on these cells, leading to apoptosis after antigen encounter, when Bim expression is up-regulated on HCV-specific CTLs, and Mcl-1 low level is unable to block Bim activity. In fact, the difference between Mcl-1 and Bim expression was significantly lower in $CD127^{low}$-expressing HCV-specific CTLs than in $CD127^{high}$ cells, and this correlated with T cell reactivity. These findings were antigen specific, because no difference in Mcl-1/Bim expression on total $CD8^+$ cells between the study groups was observed. Therefore, assuming that Mcl-1/Bim imbalance could be involved in the hyporeactivity of $CD127^{low}$- expressing HCV-specific CTLs through apoptosis activation, $CD127^{low}$ HCV-specific $CD8^+$ T cell reactivity rescue was attempted by interfering with caspase activity. Interestingly, $CD127^{low}$ HCV-specific $CD8^+$ T cell treatment with a pan-caspase inhibitor restored T cell reactivity to a similar range to the one observed in $CD127^{high}$ HCV-specific $CD8^+$ T cells from most HCV-resolved infection cases. These data suggested a link between apoptosis after TCR triggering and low CD127 expression on experienced HCV-specific CTLs during persistent infection that

could be related to Mcl-1/Bim imbalance as in chronic HBV infection [217]. Therefore, CD127- specific-CTL are prone to apoptosis due to the down-regulation of the anti-apoptotic molecule Mcl-1 and the up-regulation of the apoptosis facilitator Bim, secondary to IL-7 deprivation [217, 220]. Bearing in mind these results, at least theoretically, therapeutic apoptosis blockade could help to restore the proliferation capacity of CD127low/Mcl-1low/Bimhigh HCV-specific $CD8^+$ T cells after antigen encounter in chronic hepatitis C patients. Moreover, CD127 expression correlates inversely with the expression of several negative co-stimulatory molecules, suggesting that anergy and apoptosis are processes closely related [253], indicating that probably it is necessary to act in both of them to obtain the HCV-specific CTL response restoration. Therefore, blocking intrinsic apoptosis pathway could be one of the necessary mechanisms to improve HCV-CTL reactivity as it has been shown in other persistent hepatotropic viral infection [217]. This strategy could be a useful tool to try to improve the response to standard of care in chronic HCV patients. So far, the common calceneurin inhibitor cyclosporine-A has been shown to prevent T cell deletion through blocking Bim up-regulation [199]. Considering the *in vitro* HCV-specific CTL restoration, using the pancaspase inhibitor z-VAD-fmk, a drug as cyclosporine-A with a similar effect, but commonly used in some human diseases, could be employed in combination with anti-viral treatments to enhance the immune adaptive response.

During chronic infection, a robust viral-specific cellular response is altered, showing a pro-apoptotic phenotype due to the deprivation of IL-7

secondary to the low expression of CD127. Recently, it has been investigated that TRAF1 is a signal adapter for positive co-stimulatory receptors whose level depends on the action of IL-7 and inhibits the expression of the pro-apoptotic molecule Bim [254]. Therefore, in situations of deprivation of IL-7, action of TRAF1 could be impaired, favoring an imbalance between anti- and pro- apoptotic molecules. On the other hand, in an experimental model, IL-7 deprivation during stressing conditions leads to Mcl-1 down-regulation on T cells, conducting to T cell death that could be avoided by IL-7 treatment [230]. Consequently, strategies directed to block the pro-apoptotic effect of IL-7 deprivation should be designed to increase the effectiveness of CTL response restoration, by enhancing the TRAF1 and Mcl-1 expression level that could restore Bim/Mcl-1 balance. One of those strategies could be short-term use of FK506 could block the induction of the pro-apoptotic molecule Bim on $CD127^-$ cells [199]. This strategy could favor specific- CTL restoration during anti-viral treatments in combination with the standard of care. Another possible strategy to restore HCV-specific CTL reactivity during chronic infection could be the administration of IL-7, in order to increase the stimulation of the reduced number of IL-7R molecules on HCV-specific CTLs to modulate the balance between Bim and Mcl-1. In fact, in an animal model of cytotoxic T cell exhaustion, IL-7 treatment resulted in an increased T cell effector function and viral clearance [255].

In summary, this work suggests for the first time that CD127 phenotype modulates Bim and Mcl-1 expression on HCV-specific CTLs, and this affects T cell reactivity through apoptosis regulation. Specifically, during chronic

infection, Mcl-1/Bim imbalance could be involved in $CD127^{low}$ HCV- specific CTL hyporeactivity, but it could be overcome by pancaspase inhibition.

VII

CONCLUSION/ CONCLUSIONES

VII. Conclusion:

1. CD127 expression level on peripheral HCV-specific CTLs correlates negatively with liver damage and viral control. Therefore, during chronic HCV infection $CD127^{low}$-expresing HCV-specific CTLs are predominant.

2. CD127 expression on HCV-specific cytotoxic T cells inversely correlates with their effector functions (proliferation ability and type-1 cytokine secretion after antigen encounter).

3. Low CD127 expression level up-regulates pro-apoptotic molecule-Bim after antigen encounter and down-regulates anti-apoptotic molecule Mcl-1 in HCV specific $CD8^+$ T cells during chronic HCV infection.

4. The balance between pro- and anti-apoptotic molecules is critical for HCV specific CTL survival. The CD127 down-regulation modulates Bim and Mcl-1 balance on HCV-specific CTLs and this affect to T cell reactivity through apoptosis induction.

5. Consequently, Mcl-1/Bim imbalance could be the reason for the deletion of HCV-specific T cells, but it could be overcome by apoptosis blockade.

VII. Conclusiones:

1. El nivel de expresión de CD127 en los LTC VHC-específicos se correlaciona de forma negativa con el daño hepático y el control viral. Por tanto, los LTC VHC-específicos con baja expresión de CD127 son predominantes durante la infección crónica por VHC.

2. La expresión de CD127 en los LTC VHC-específicos se correlaciona de forma inversa con las funciones efectoras de estas células (capacidad de proliferación y de secrecion de citoquinas tipo1).

3. Un nivel bajo de expresión de CD127 en las células T $CD8^+$ VHC-específicas durante la infección crónica por VHC provoca la sobre-expresión de Bim tras el encuentro antigénico y la disminución de la expresión de Mcl-1.

4. El equilibrio entre moléculas pro- y anti-apoptóticas es crítico para la supervivencia de los LTC VHC-específicos. La disminución de la expresión de CD127 en los LTC VHC-específicos modula el equilibrio entre Bim y Mcl-1 y esto afecta a la relatividad de estas células a través de la inducción de la apoptosis.

5. En consecuencia, el desequilibrio entre Mcl-1/Bim podría ser la razón de la eliminación de los LTC VHC-específicos aunque esto puede ser evitado mediante el bloque de la apoptosis.

VIII

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2.- JR Larrubia, **MU Lokhande**, S García-Garzón, J Miquel, A González-Praetorius, E Sanz-de-Villalobos. Peripheral HCV-specific cytotoxic response detection at week 12 of pegylated-interferon alfa 2b plus ribavirin treatment for chronic HCV infection correlatos with sustained virologic response development. **EASL 2013 Amsterdam. J Hepatol 2013**; 58(S1): 144. ISSN: 0168-8278

3.- JR Larrubia, **MU Lokhande**, T Parra-Cid, J Miquel, S García-Garzón, A González-Praetorius, E Sanz-de-Villalobos. $CD127^{low}$ HCV-specific cytotoxic T cell hyporeactivity during persistent infection depends on Mcl-1/Bim ratio. **EASL 2012 Barcelona. J Hepatol 2012**; 56(S2): 331-2. ISSN: 0168-8278

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Persistent hepatitis C virus (HCV) infection impairs HCV-specific cytotoxic T cell reactivity through Mcl-1/Bim imbalance due to CD127 down-regulation

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SUMMARY. In persistent hepatitis C virus (HCV) infection, HCV-specific cytotoxic T lymphocyte (CTL) reactivity is impaired and this affects HCV control. Interleukin-7 receptor (CD127) expression on these cells could regulate CTL reactivity through Mcl-1/Bim balance modulation. Bim is a pro-apoptotic molecule blocked by the action of Mcl-1. Mcl-1/Bim expression and T cell reactivity on HCV-specific CTLs were compared according to CD127 phenotype. Peripheral blood lymphocytes (PBL) from HLA-A2$^+$ HCV$^+$ patients were obtained. HCV-specific CTLs were visualized by staining PBL with anti-CD8 and HLA-A2/peptide pentameric complexes (pentamer). Mcl-1/Bim/CD127 phenotype of HCV-specific CTLs was tested by staining detectable CD8$^+$/pentamer$^+$ cells with anti-Mcl-1/Bim/CD127 antibodies. HCV-specific CTL proliferation ability after specific *in vitro* challenge was tested in the presence and absence of pancaspase inhibitor z-VAD-fmk. All stained cells were analysed by flow cytometry. CD127^{low}-expressing HCV-specific CTLs associated with high HCV viraemia, while CD127^{high} correlated with undetectable viral loads ($P < 0.001$). Directly *ex vivo*, pentamer$^+$ cell frequency was similar according to CD127 expression level. Nevertheless, CD127^{low} pentamer$^+$ cell proliferation after specific *in vitro* challenge was impaired ($P < 0.05$), although this was corrected by z-VAD-fmk treatment ($P < 0.05$). Mcl-1 expression was low directly *ex vivo* ($P < 0.01$), and Bim was up-regulated after antigen encounter ($P < 0.05$) of CD127^{low} pentamer$^+$ cells. The *ex vivo* difference between Mcl-1 and Bim expression on pentamer$^+$ cells correlated positively with CD127 expression level ($P < 0.001$) and with pentamer$^+$ cell reactivity ($P < 0.05$). In summary, a low *ex vivo* Mcl-1 expression and Bim up-regulation after antigen encounter are involved in CD127^{low} HCV-specific CTL hyporeactivity during chronic infection, but it can be overcome by apoptosis blockade.

Keywords: Bim, CD127, HCV-specific CTL, Mcl-1, T cell reactivity, z-VAD-fmk.

INTRODUCTION

Hepatitis C virus (HCV) infection progresses to a chronic disease in most cases and is a major cause of hepatic cirrhosis and hepatocellular carcinoma [1]. HCV-specific cytotoxic T lymphocytes (CTLs) play an important role in HCV control during acute infection [2]. Unfortunately, during chronic infection, the reactivity of these cells is impaired after antigen encounter, preventing their effector functions [3] and allowing the development of persistent liver inflammation [4]. After natural or treatment-induced HCV control, a peripheral population of HCV-specific CTLs is maintained. These cells are characterized by IL-7 receptor (CD127) expression [5] and are able to control residual HCV traces after clinical disease control [6] and to respond faster to a new infection [7].

CD127 plays an essential role in mature lymphocyte survival by counteracting the induction of apoptosis after antigen encounter through regulation of some of the B cell lymphoma 2 (Bcl-2) proteins, enhancing IL-2 secretion and life span [8]. Therefore, HCV could modulate CD127 expression on HCV-specific CTLs to impair the quality of the adaptive immune response by IL-7 deprivation, as a survival strategy [5,9]. Passive T cell death, or death by cytokine deprivation, is controlled by members of the Bcl-2 family, such as Bim and Mcl-1. In experimental models, Bim carries

Abbreviations: ALT, alanine aminotransferase, Bcl-2, B cell lymphoma 2, CTL, cytotoxic T lymphocyte; HCV, hepatitis C virus; IFN, γ-interferon; IQR, interquartile range; MFI, mean fluorescence intensity; PBL, peripheral blood lymphocytes; TGF, transforming growth factor.

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out a pro-apoptotic role in intrahepatic T cells that is counteracted by the Mcl-1 action [10,11]. Interestingly, a role of Bim level on HCV-specific CTL reactivity, according to CD127 expression, has been suggested recently by our group but this pathway has not been described in depth [5]. In this article, the correlation between CD127 expression on HCV-specific CTLs and the Mcl-1/Bim phenotype and their effect on T cell reactivity were analysed.

PATIENTS, MATERIAL AND METHODS

Patients

In this cross-sectional study, peripheral blood lymphocytes (PBL) from 24 consecutive HLA-A2$^+$ HCV$^+$ genotype-1 subjects, previously known to have directly *ex vivo* HCV-specific CTLs against one or two different HCV-NS3 immunodominant epitopes, were obtained between January 2007 and August 2011 at Guadalajara University Hospital, Spain. From these cases, 33 different HCV-specific CTL populations were isolated; 20 of them specific against $NS3_{1406–1415}$ and another 13 specific against $NS3_{1073–1081}$. These samples were split into two groups according to CD127 expression level on HCV-specific CTLs. The patients' clinical and virological features, regarding these two CD127 groups, are shown in Table 1. Other causes of liver disease were excluded. In all recruited patients, serum samples to test HCV viral load and alanine aminotransferase (ALT) level and heparinized blood samples to obtain PBL were taken. HCV genotype data were obtained from the patients' clinical records. The study protocol was approved by the Regional Ethical Committee, and patients gave written informed consent before enrolment.

Table 1 Clinical and virological features of the HCV-specific CTL samples tested in the study

	CD127^{high} *N* = 14	CD127^{low} *N* = 19	*P* value*
Age (years old)	44 (4)	46 (15)	NS
Sex (M/F) (%)	78/22	77/23	NS
Diagnosis (%)			
SVR	86	5	<0.001
CHC	14	95	
Time of evolution (years)	24 (6)	29 (24)	NS
ALT (IU/mL)	40 (31)	72 (82)	<0.001
HCV viral load (IU/mL) (log scale)	1.6 (0.8)	6.06 (0.96)	<0.001
Genotype 1a/1b (%)	30/70	55/45	NS
Epitopes tested ($NS3_{1406}$/$NS3_{1073}$) (%)	73/26	54/46	NS

CHC, chronic hepatitis C; CTL, cytotoxic T lymphocyte; F, female; HCV, hepatitis C virus; M, male; *N*, number of samples; NS, nonsignificant; SVR, sustained virologic response.
*Mann–Whitney *U* test.

Tissue typing

Screening for the HLA-A2 haplotype was performed by staining PBL with fluorescein isothiocyanate (FITC)-conjugated anti-HLA-A2 monoclonal antibody (mAb; BD Bioscience, Franklin Lakes, NJ, USA) and subsequent flow cytometric analysis.

Virological assessment

Anti-HCV was determined by Ortho HCV version 3.0 ELISA Test (Ortho Diagnostic System, Raritan, NJ, USA). HCV-RNA load was assessed by a quantitative real-time-PCR assay with a lower detection limit of 40 IU/mL (Cobas Amplicor HCV Monitor 2.0; Roche, Indianapolis, IN, USA). HCV genotype was tested by InnoLiPPA HCV II assay (Innogentics Inc, Alpharetta, GA, USA).

Synthetic peptides and pentamers

A2-restricted peptides corresponding to the genotype-1 $NS3_{1406–1415}$ region (KLVALGINAV), $NS3_{1073–1081}$ region (CINGVCWTV) and Phycoerithrin-conjugated (PE-conjugated) HLA-A2 pentameric complexes (pentamer) loaded with the same two NS3 peptides were purchased from ProImmune (Oxford, UK).

CD8$^+$/pentamer$^+$ cell quantification

Peripheral blood lymphocytes were purified from heparinized blood samples through a Ficoll-Hypaque gradient. To quantify HCV-specific CD8$^+$ cells, 0.5–1 × 10^6 PBL were incubated for 10 min at 37 °C with 1 μg of PE-labelled pentamers in RPMI 1640 plus 10% fetal calf serum (FCS). Cells were washed in phosphate buffer saline plus 1% FCS and then incubated at 4 °C for 20 min with saturating concentrations of directly conjugated anti-CD8-Pe-Cy5 (Cy) mAb (RD Systems, Minneapolis, MN, USA). Subsequently, after washing, stained cells were analysed on a Becton Dickinson FACS® using CELLQuest™ software (BD Biosciences, San Jose, CA, USA). HCV-specific CTL quantification was expressed as the percentage of CD8$^+$/pentamer$^+$ cell out of the total CD8$^+$ cells. The lowest percentage at which pentamer-binding CD8$^+$ cells could be detected as a separate cell population was 0.018% out of the total CD8$^+$ cells.

CD127 phenotypic analysis on CD8$^+$/pentamer$^+$ cell

Peripheral blood lymphocytes were stained with PE-pentamers plus anti-CD8-Cy and FITC-conjugated anti-CD127

(Ebioscience, Hatfield, UK) mAbs as above. Subsequently, stained cells were analysed by flow cytometry. Isotype-matched control mAbs were used to set the markers. CD127 phenotype was quantified on pentamer$^+$ cells as the mean fluorescence intensity (MFI). Based on our previous report [5], CD8$^+$/Pentamer$^+$ cells were classified into two groups according to the anti-CD127-FITC mAb MFI level (CD127^{high} group: MFI ≥ 20 units; CD127^{low} group: MFI < 20 units).

Production of T cell lines

Peripheral blood lymphocytes were re-suspended at a concentration of 1×10^6/mL in complete medium plus 10% FCS. Cells were stimulated with 1 μM of one of the two NS3-specific peptides, NS3$_{1406–1415}$ and NS3$_{1073–1081}$, in a 96-well plate. Recombinant IL-2 (25 IU/mL) (RD Systems) was added at the beginning and on day 2 of culture, and cells were analysed after a total of 10 days of culture. PBL were also cultured in the presence of either the pancaspase inhibitor zVAD-fmk (50 μM) (BD Bioscience, San Jose, CA, USA), or β-galactosidase (2 μg/mL) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as control. Expansion was considered positive when a pentamer-binding CD8$^+$ cell population, in a cluster shape, bigger than base line and higher than 0.2% of the total CD8$^+$ cells was observed after specific stimulation for 10 days.

Intracellular Bim/Mcl-1 staining

Pentamer$^+$/CD8$^+$ T cells were surface-stained with PE-pentamers and CD8-Cy mAb as above. After a further wash, cells were subjected to intracellular staining using Cytofix-Cytoperm (BD Bioscience) to permeabilize and fix cells according to the manufacturer's instructions, followed by staining with Bim unconjugated rabbit anti-human polyclonal Ab (Cell Signaling, Boston, MA, USA), Mcl-1 unconjugated rabbit anti-human mAb (Epitomics, Burlingame, CA, USA) and its matched controls, plus the secondary mAb, goat anti-rabbit IgG Alexa-Fluor 488 (Invitrogen, Grand Island, NY, USA). Finally, cells were washed twice and analysed by flow cytometry. Data were expressed as Alexa-Fluor 488 MFI on gated pentamer$^+$/CD8$^+$ cells. The absolute value of Alexa-Fluor 488 MFI for Mcl-1 and Bim staining and the difference between these two values were compared according to CD127 expression level, and correlated with pentamer$^+$/CD8$^+$ cell reactivity. The cut-off for Mcl-1/Bim positive staining was set according to the mean plus 2 standard deviations of the MFI observed with the matched control stainings.

Intracellular γ-interferon (IFN) staining

Peripheral blood lymphocytes were in vitro challenged with NS3$_{1406–1415}$ peptide, for 5 h at 37 °C seeded at 1×10^6 cells/mL in RPMI 1640, plus 10% FCS, in the presence of Brefeldin A (10 μg/mL; Sigma-Aldrich Inc, St. Louis, MO, USA), during the last 4 h of incubation. Cells were washed and surface-stained with PE-pentamers as above. After a further wash, cells were subjected to intracellular staining using Cytofix-Cytoperm (BD Bioscience) to permeabilize and fix cells, followed by staining with FITC-conjugated anti-IFN mAb and its isotype-matched control (Pharmingen BD, San Jose, CA). Finally, cells were washed twice and analysed by flow cytometry. γ-IFN data are shown as the percentage of γ-IFN-producing pentamer$^+$ cells out of the total pentamer$^+$ cells.

Statistical analysis

Continuous and categorical variables are summarized as median plus interquartile range (IQR) and as frequency distribution, respectively. Mann–Whitney *U*, Wilconxon and Spearman tests were employed where appropriate. All the tests were two-tailed and with a significance level of $P < 0.05$.

RESULTS

Viral replication and liver damage according to CD127 expression on pentamer$^+$ cells

Thirty-three different pentamer$^+$ populations from 24 HLA-A2$^+$ HCV$^+$ genotype-1 patients, with directly *ex vivo* detectable pentamer$^+$ cells, were recruited for this study. In 20 cases, NS3$_{1406–1415}$ pentamer$^+$ cells were observed, while this was the case in 13 NS3$_{1073–1081}$ pentamer$^+$ cell patients. All the phenotypic results, obtained with the two different pentamers tested, were pooled into the same variable for statistical analysis. Samples were split into two groups according to CD127 expression level, as shown in Patients, Material and Methods. Both CD127 groups were similar with respect to patient's age, disease evolution time and sex distribution (Table 1). CD127^{high} samples belonged to patients with undetectable or low viral load, and low ALT level, while CD127^{low} HCV-specific CTLs associated with high viral load and high ALT serum concentration ($P < 0.001$) (Table 1 and Fig. 1). In fact, most patients with CD127^{high}-expressing pentamer$^+$ cells were sustained virologic responders, tested at least 6 months after finishing standard anti-HCV treatment, while CD127^{low}-expressing cells were mainly present in naïve chronic hepatitis C patients (Table 1). The CD127 expression on pentamer$^+$ cells in the CD127^{low} group was almost three times lower (MFI, 13.90; IQR, 8.37) than in the CD127^{high} group (MFI, 34.59; IQR, 10.02) ($P < 0.001$; Figs 1 and 4) and similar to the MFI observed with the isotypic control staining (MFI, 5.60; IQR, 4.30) ($P > 0.05$). This difference was antigen specific, because in both groups a similar CD127 expression on total CD8$^+$ cells was observed (Fig. 1).

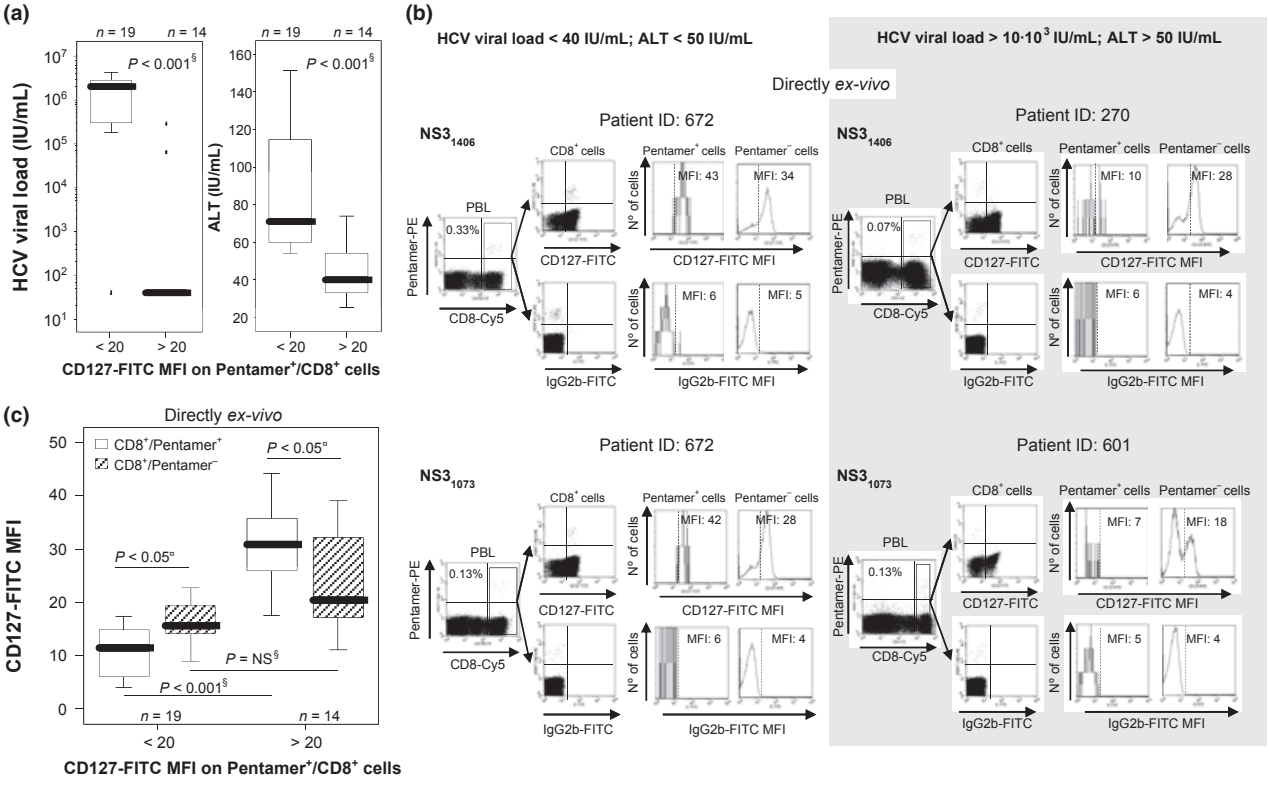

Fig. 1 Liver damage and viral control according to CD127 expression on hepatitis C virus (HCV)-specific cytotoxic T cells. (a) Box plots showing serum ALT (IU/mL) and viral load (IU/mL) levels according to the CD127 expression on HCV-specific cytotoxic T cells (pentamer$^+$/CD8$^+$ cells). (B) Representative FACS® dot plots and MFI histograms of peripheral T cells stained with labelled mAb against CD8 and CD127 and with pentameric HLA-A2/peptide PE-labelled complexes against $NS3_{1406}$ and $NS3_{1073}$ HCV epitopes from cases with different degrees of liver damage and viral control. The figure on the upper left corner of the FACS® dot plots for pentamer/CD8 staining shows the frequency of pentamer$^+$ cells out of the total CD8$^+$ cells. FACS® histograms are gated on pentamer$^+$ and pentamer$^-$ CD8$^+$ cells. The dashed line in the histograms represents the upper limit of the staining with the isotypic control. (c) Box plots showing the directly *ex vivo* CD127 MFI on total CD8$^+$ and pentamer$^+$ cells in the two groups of the study. §Mann–Whitney *U* test. ¤Wilconxon test. ID, patient identification; MFI, mean fluorescence intensity; *n*, number of cases; PBL, peripheral blood lymphocytes. *Outlier values.

Reactivity of pentamer$^+$ cells according to CD127 expression level

To test whether a low CD127 expression level could affect HCV-specific CTL reactivity after antigen encounter because of apoptosis activation, in ten cases of each group the proliferation ability of pentamer$^+$ cells after specific *in vitro* challenge, in the presence of either a pancaspase inhibitor (z-VAD-fmk) or β-galactosidase as control, was analysed. Directly *ex vivo*, the peripheral frequency of pentamer$^+$ cells out of the total CD8$^+$ cells was very low and similar in both groups; CD127^{low}: 0.05% (IQR, 0.09) and CD127^{high}: 0.04% (IQR, 0.05) ($P > 0.05$, Figs 2 and 3). Nevertheless, the frequency of pentamer$^+$ cells, after stimulation with the specific peptides for 10 days without z-VAD-fmk treatment, was higher in the CD127^{high} than in the CD127^{low}-expressing pentamer$^+$ cells. The median frequency of pentamer$^+$ cells was 1.7% (IQR, 6.77) in the CD127^{high} group while it was only 0.003% (IQR, 2.9) in CD127^{low} group ($P < 0.05$, Figs 2 and 3). Moreover, considering a positive proliferation as a cluster of pentamer$^+$ cells higher than 0.2% out of the total CD8$^+$ cells, only in 30% of the CD127^{low} cases proliferation was observed, while 80% of samples did proliferate in the CD127^{high} group ($P < 0.05$, Fig. 3). Nevertheless, CD127^{low}-expressing pentamer$^+$ cells improved in proliferation ability after specific stimulation in the presence of z-VAD-fmk treatment (1.39%; IQR, 13.2) with respect to the observed expansion without this pancaspase inhibitor ($P < 0.05$, Figs 2 and 3), reaching a similar expansion level to the CD127^{high} group (4.5%; IQR, 6.25) ($P > 0.05$, Figs 2 and 3). z-VAD-fmk treatment also raised nonsignificantly the proliferation capacity of CD127^{high}-expressing cells ($P > 0.05$, Figs 2 and 3). Furthermore, in the CD127^{low} group, the percentage of experiments with pentamer$^+$ cell proliferation increased after z-VAD-fmk treatment up to 80% ($P < 0.05$) (Fig. 3), giving a similar value to the one

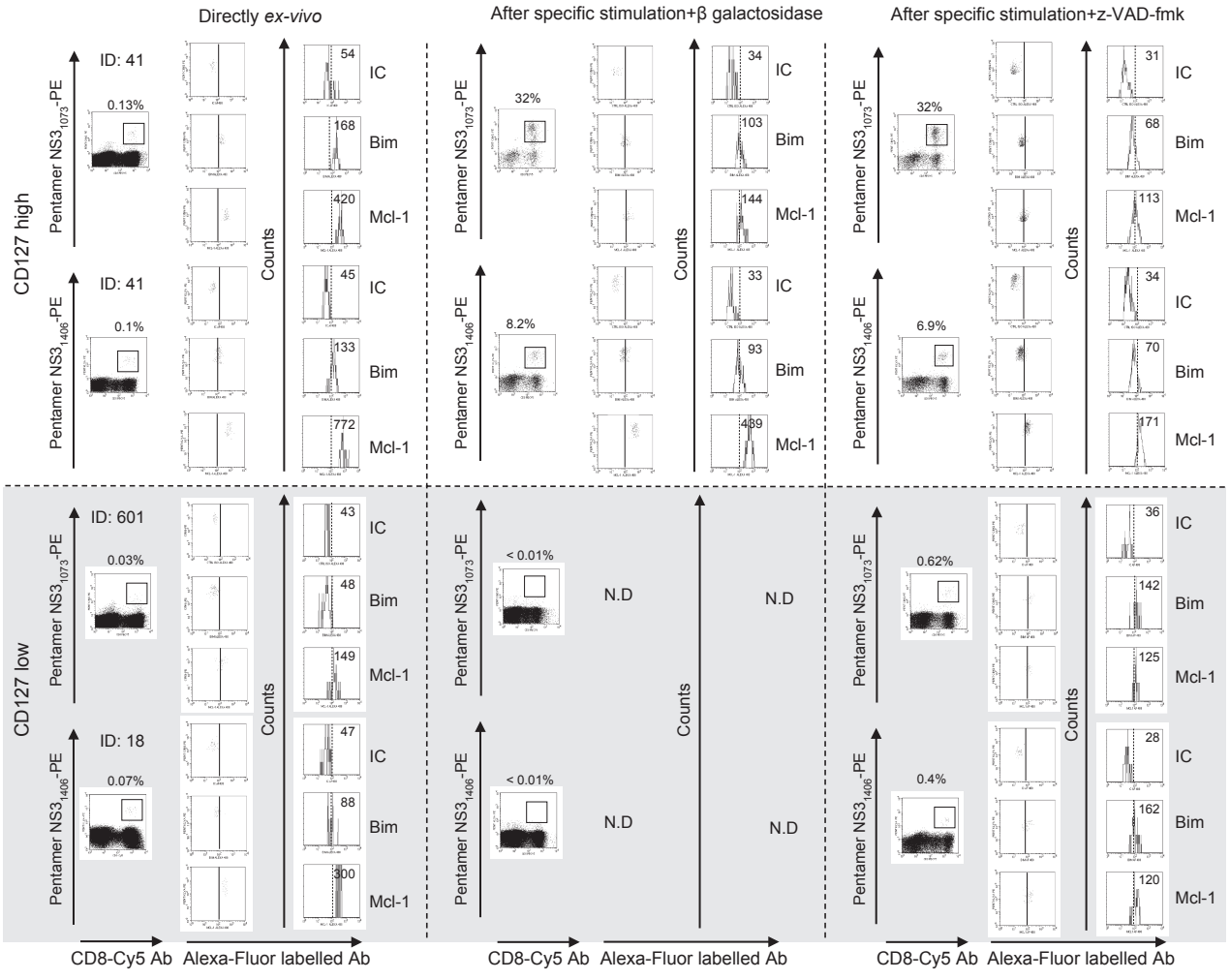

Fig. 2 Bim and Mcl-1 expression directly *ex vivo* and after specific stimulation on peripheral CD8$^+$/pentameter$^+$ cells according to CD127 level. Representative FACS® dot plots and mean fluorescence intensity (MFI) histograms showing the Mcl-1 and Bim expression on peripheral CD8$^+$/pentamer$^+$ cells, directly *ex vivo* and after specific *in vitro* challenge, with regard to the CD127 expression level on those cells. Peripheral blood lymphocytes were subjected to specific *in vitro* challenge in the presence of z-VAD-fmk and *β*-galactosidase as control. The figure on the top of the dot plots represents the frequency of pentamer$^+$ cells out of the total CD8$^+$ cells. The figure in the upper right corner in the histogram plot represents the MFI for Bim and Mcl-1 staining. The continuous and dashed line in the dot plots and histograms represent the cut-off point for positive staining according to the negative control. IC, isotypic control; ID, patient identification; N.D., not done because of lack of pentamer$^+$/CD8$^+$ proliferation after specific stimulation.

observed in the CD127^{high} group ($P > 0.05$) (Fig. 3). Interestingly, CD127 expression decreased significantly after specific *in vitro* stimulation of CD127^{high}-expressing pentamer$^+$ cells ($P < 0.001$) to a similar level (MFI, 8.05; IQR, 5.90) to the one observed directly *ex vivo* in the CD127^{low} group (Figs 3 and 4).

γ-Interferon production by pentamer$^+$ cells according to CD127 phenotype

Directly *ex vivo* γ-IFN-producing pentamer$^+$ cells were analysed in 11 cases (5 CD127^{high} and 6 CD127 ^{low}). The frequency of γ-IFN-producing pentamer$^+$ cells was lower in the CD127^{low} (40%; IQR, 32) than in the CD127^{high} group (61%; IQR, 13.5) ($P < 0.05$, Fig. 3).

Mcl-1 and Bim expression according to CD127 phenotype

After the demonstration of impaired reactivity on CD127^{low} pentamer$^+$ cells with regard to apoptosis induction, two Bcl-2 family protein phenotypes, involved in the extrinsic apoptosis pathway, were analysed on 28 populations of pentamer$^+$ cells to see whether those phenotypes could explain the different T cell reactivity according to CD127 level. Bim and Mcl-1 MFI on gated CD8$^+$ pentamer$^+$ cells, stained with Alexa-Fluor 488 labelled Ab against these two

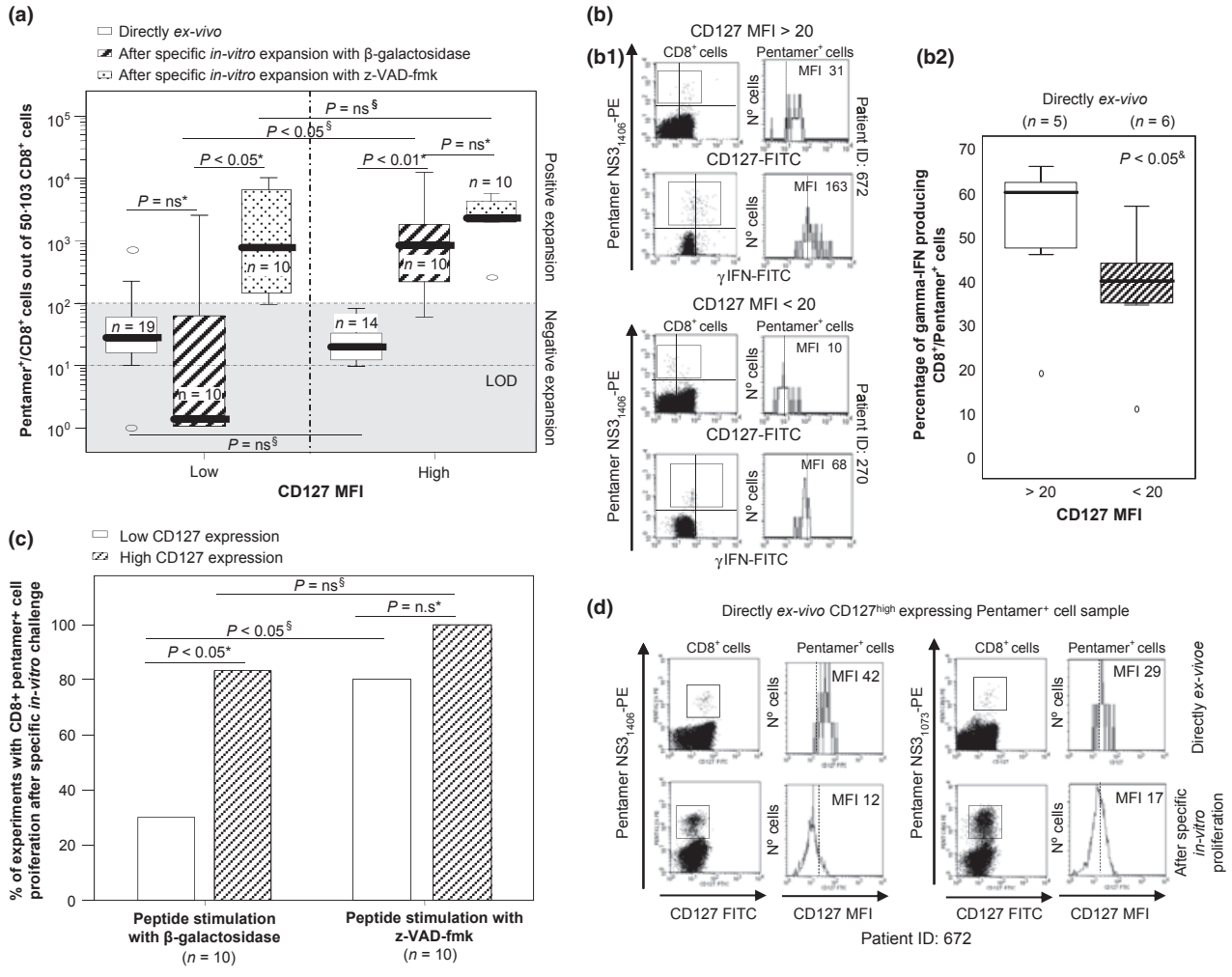

Fig. 3 Reactivity of $CD8^+$/pentamer$^+$ cells according to CD127 expression level. (a) Box plots showing the frequency of $CD8^+$/pentamer$^+$ cells directly *ex vivo* and after specific *in vitro* challenge in the presence of either z-VAD-fmk or *β*-galactosidase as control in high and low CD127-expressing pentamer$^+$ cells. (b) (b.1) Representative FACS® dot plots and mean fluorescence intensity (MFI) histograms showing directly *ex vivo* *γ*-interferon (IFN) production on pentamer$^+$ cells according to CD127 expression. (b.2) Box plots showing the percentage of IFN-producing pentamer$^+$ cells, in relation to CD127 expression level. The figure in the upper right corner in the histogram plot represents the MFI for IFN staining. (c) Bar plots showing the percentage of experiments with $CD8^+$/pentamer$^+$ cell proliferation after specific *in vitro* challenge in the presence of either z-VAD-fmk or *β*-galactosidase as control, with regard to CD127 expression level. Positive proliferation was considered when more than 0.2% of $CD8^+$/pentamer$^+$ cells in a clear cluster were observed after specific stimulation. (d) Representative FACS® dot plots and MFI histograms from $CD127^{high}$-expressing pentamer$^+$ cells, with CD127 expression on $CD8^+$/pentamer$^+$ cells directly *ex vivo* and after specific *in vitro* challenge. *Wilconxon test. §Mann–Whitney *U* test. LOD, limit of detection; MFI, mean fluorescence intensity; *n*, number of cases; n.s., nonsignificant; O, outliers.

molecules, were calculated. A value was considered positive when it was higher than the MFI mean plus 2 standard deviations obtained from the pool of pentamer$^+$ cell staining with the isotypic Ab control (Cut-off level: 90 unit of fluorescence). Directly *ex vivo*, Bim expression on peripheral pentamer$^+$ cells was similar on $CD127^{low}$ pentamer$^+$ (MFI, 79.19; IQR, 117.60) and on $CD127^{high}$-expressing cells (MFI, 156.20; IQR, 136.01) ($P > 0.05$, Figs 2 and 4). Nevertheless, directly *ex vivo* Mcl-1 expression was significantly lower on $CD127^{low}$ pentamer$^+$ (MFI, 172.80; IQR, 119.57) than on $CD127^{high}$-expressing cells (MFI, 278.92; IQR, 266.65) ($P < 0.01$, Figs 2 and 4). These data translated into a significant positive correlation between CD127 expression and Mcl-1 level on peripheral pentamer$^+$ cells ($r = 0.426$, $P < 0.05$), while no significant correlation between CD127 and Bim expression was observed ($P > 0.05$) (Fig. 4). These findings were antigen specific because in both study groups a similar directly *ex vivo* Mcl-1/Bim expression on total $CD8^+$ cells was observed (Fig. 4). Bearing in mind that Mcl-1 could counteract Bim action, the excess of Mcl-1 expression over

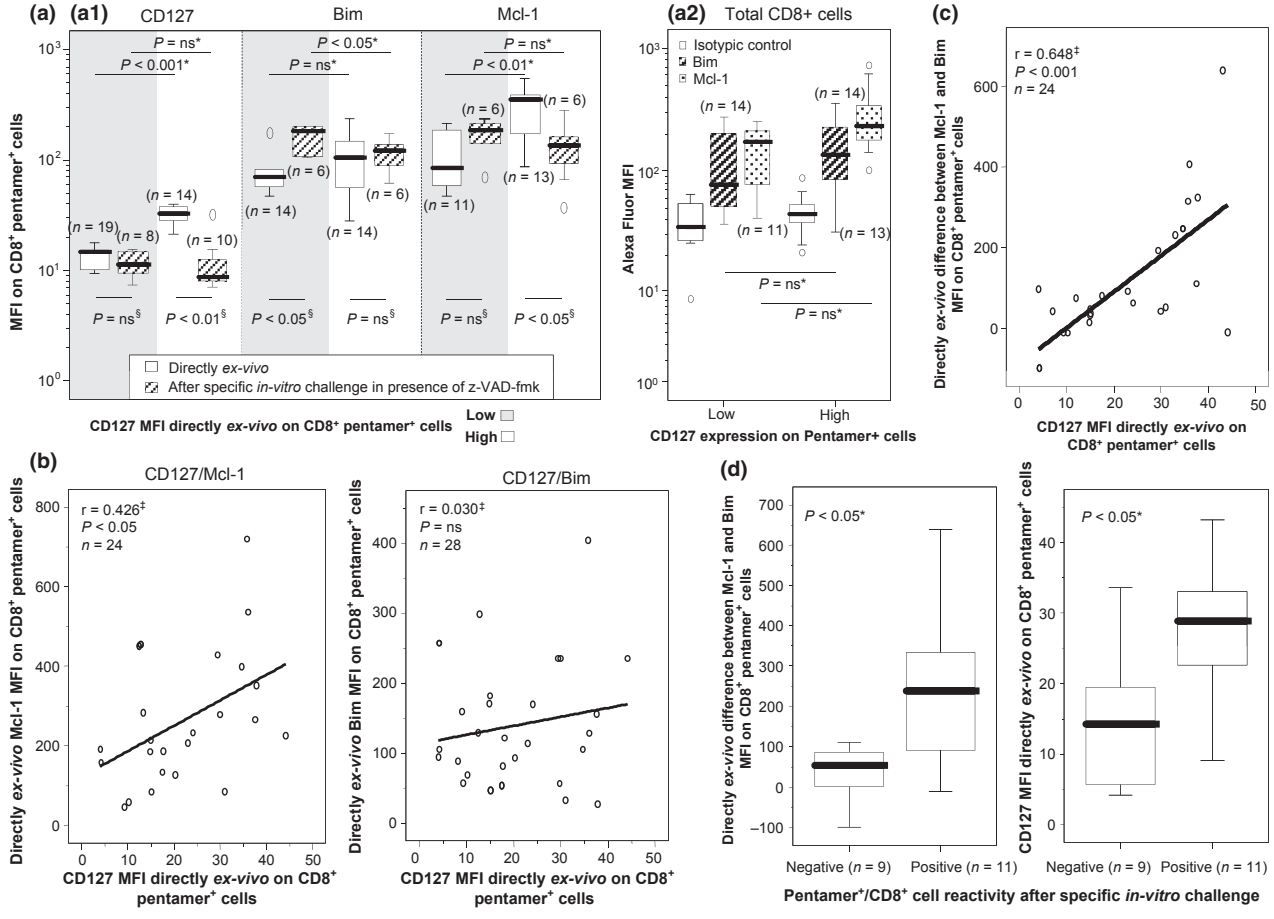

Fig. 4 Mcl-1 and Bim expression on CD8$^+$/pentamer$^+$ cells according to CD127 expression level and their relationship with CD8$^+$/pentamer$^+$ cell reactivity after antigen encounter. (a) (a.1) Box plots showing the MFI for CD127, Mcl-1 and Bim staining on peripheral CD8$^+$/pentamer$^+$ cells directly *ex vivo* and after specific *in vitro* challenge, blocking apoptosis, according to CD127 expression level. (a.2) Box plots describing the directly *ex vivo* Mcl-1/Bim expression on total CD8$^+$ cells in the two groups of the study. (b) Scatter plots showing the correlation between CD127 expression on CD8$^+$/pentamer$^+$ cells and Mcl-1/Bim expression on peripheral CD8$^+$/pentamer$^+$ cells. (c) Scatter plot showing the positive correlation between the Mcl-1/Bim expression subtraction and CD127 expression level on peripheral CD8$^+$/pentamer$^+$ cells. (d) Box plots describing the CD127 expression level and Mcl-1/Bim expression difference on peripheral CD8$^+$/pentamer$^+$ cells with regard to their reactivity after antigen encounter. §Wilconxon test. *Mann–Whitney *U* test. ‡Spearman's correlation coefficient. MFI, mean fluorescence intensity; *n*, number of cases; n.s., nonsignificant; O, outliers.

Bim level on pentamer$^+$ cells was calculated by subtracting Mcl-1 Alexa-Fluor 488 MFI level to the MFI observed for Bim staining. This calculated variable showed a high significant positive correlation with CD127 expression on pentamer$^+$ cells ($r = 0.648$, $P < 0.001$, Fig. 4). In fact, the difference between Mcl-1 and Bim expression was almost four times higher on CD127^{high}-expressing pentamer$^+$ cells (difference, 110.74; IQR, 267.57) than on CD127^{low} cells (difference, 32.50; IQR, 99.54) ($P < 0.01$). The excess of Mcl-1 expression, regarding Bim levels, was also higher in pentamer$^+$ cells with proliferation ability after antigen encounter (difference, 239.57; IQR, 308.68) than in cells without reactivity (difference, 52.71; IQR, 102.60) ($P < 0.05$, Fig. 4), showing a similar profile to the CD127 level, as far as expansion capacity was concerned. Thus, pentamer$^+$ cells with proliferation ability expressed a higher CD127 level (MFI, 29.36; IQR, 22.85) and a higher Mcl-1/Bim expression difference than pentamer$^+$ cells without expansion potential, characterized by a low CD127 expression (MFI, 16.80; IQR, 22.85) ($P < 0.05$, Fig. 4).

To further analyse whether T cell receptor (TCR)/HLA-I-peptide engagement could influence Mcl-1/Bim imbalance, according to CD127 expression level, the Mcl-1/Bim/CD127 phenotype, after specific *in vitro* challenge in the presence of z-VAD-fmk, was also tested and compared between both CD127 expression groups. To study Bim and Mcl-1 expression after Ag encounter on CD127^{low}-expressing pentamer$^+$ cells, z-VAD-fmk was used to ensure the proliferation of these cells. After CD127^{high}-expressing pentamer$^+$ cell specific antigen stimulation, CD127 expression was down-regulated

($P < 0.01$) to a similar level to the one observed on $CD127^{low}$ cells, as previously commented, in association with Mcl-1 down-regulation (MFI, 134.57; IQR, 106.04) ($P < 0.05$), which reached the level described on $CD127^{low}$ cells but without Bim expression change (MFI, 121.04; IQR, 79.04) ($P > 0.05$) (Figs 2 and 4). On the other hand, in $CD127^{low}$-expressing pentamer$^+$ cells, CD127 expression was maintained at low levels after specific *in vitro* challenge (MFI, 11.28; IQR, 8.26) ($P > 0.05$), and Mcl-1 expression was not modified (MFI, 199.23; IQR, 177.95), but interestingly, Bim expression was significantly up-regulated (MFI, 191.08; IQR, 147.87) ($P < 0.05$) to a significantly higher level than the one expressed by $CD127^{high}$-expressing pentamer$^+$ cells ($P < 0.05$) (Figs 2 and 4). These data suggest that Bim expression can be up-regulated only after TCR triggering in cells with low CD127 expression.

DISCUSSION

Hepatitis C virus-specific CTLs are essential effector tools to control HCV infection, but they are anergic and prone to apoptosis during persistent infection [5,12]. This phenomenon may be related to a high activation of these cells during persistently elevated viraemia during chronic HCV infection [13,14]. In fact, upon continued antigenic stimulation, $CD8^+$ T cells lose their effector function and are selectively deleted, resulting in failure to achieve viral clearance, and this fact correlates with CD127 down-regulation [15]. This could be a physiological strategy by the host to avoid immune-mediated injury during chronic infection. Therefore, in persistent HCV infection, peripheral HCV-specific CTLs could acquire an exhausted phenotype to avoid a harmful immune response. Subsequently, these cells cannot withstand further activation in the liver after antigen re-encounter, leading to selective T cell deletion.

The procedure of T cell death during chronic viral infection is determined by a carefully balanced and complex group of pro- and anti-apoptotic proteins of the Bcl-2 family, such as Bim and Mcl-1 [10]. Bim is clearly involved in intrahepatic-specific CTL apoptosis in animal models [16]. Bim is thought to act principally by activating Bax, permitting mitochondrial release of cytochrome *c*, which activates caspases, ultimately leading to cell death [10]. Expression of Bim is up-regulated in human T cells in response to TCR triggering by the protein kinase C (PKC) and calcineurin pathways [17]. Nevertheless, there are other mechanisms involved in Bim up-regulation during chronic infection, such as the effect of certain cytokines. IL-7 withdrawal could induce Bim-mediated apoptosis. In fact, in a persistent viral infection animal model, Bim-mediated apoptosis correlates with low IL-7 receptor expression on specific T cells [18]. Transforming growth factor (TGF)-β is also involved in Bim-mediated apoptosis [19]. Therefore, all these data indicate that Bim action is not only modulated through TCR stimulation, but also by other regulatory mechanisms such as either IL-7 withdrawal or TGF-β induction. Interestingly, persistent HCV infection is characterized by continuous TCR triggering and CD127 down-regulation on HCV-specific CTLs [12], which could favour, so far, Bim up-regulation. In our work, Bim was up-regulated on $CD127^{low}$-expressing HCV-specific CTLs but not on $CD127^{high}$ cells after antigen encounter, suggesting that TCR triggering can only lead to Bim up-regulation in the absence of IL-7 stimulation on HCV-specific CTLs. Nevertheless, Bim level is not enough to lead to T cell apoptosis. T cell death also depends on the anti-apoptotic protein expression level, regulated by cytokines, such as IL-7. Regarding this, the Bim pro-apoptotic effect is blocked by the action of the Bcl-2 family of anti-apoptotic proteins such as Mcl-1 and Bcl-2 [20,21]. Interestingly, in an experimental model, IL-7 deprivation during stressing conditions leads to Mcl-1 down-regulation on T cells, leading to T cell death that could be avoided by IL-7 treatment [22]. In agreement with this experimental model, our *ex vivo* data show that during persistent HCV infection the predominant peripheral population of HCV-specific CTLs displays a $CD127^{low}$/$Mcl\text{-}1^{low}$ phenotype that remains low after antigen encounter. All in all, these Mcl-1/Bim data could mean that during chronic HCV infection, experienced $CD127^{low}$/$Mcl\text{-}1^{low}$-expressing T cells attracted to the liver would up-regulate Bim expression after antigen encounter because of the absence of IL-7 stimulus, and subsequently Bim would be released freely to activate Bax, because of the low level of anti-apoptotic proteins such as Mcl-1. Consequently, CD127 level would play a central role in HCV-specific $CD8^+$ T cell apoptosis by regulation of Mcl-1 expression *in vivo* and by Bim modulation after antigen encounter. This theory could explain why $CD127^{low}$ HCV-specific $CD8^+$ T cell reactivity from chronic hepatitis C patients is impaired, and theoretically this could be overcome by apoptosis blockade. To test this hypothesis, the correlation between CD127 phenotype, T cell reactivity and Bim/Mcl-1 phenotype on HCV-specific CTLs after apoptosis blockade was tested in this study.

In this work, directly *ex vivo* HCV-specific CTLs from a cohort of patients with different degrees of viral control were obtained. In patients with either low or undetectable viral load, a population of peripheral $CD127^{high}$-expressing cells able to proliferate and to produce γ-IFN after antigen encounter was observed. Theoretically, this population could control HCV traces or be ready for a rapid response in the case of re-infection [6,7]. These cells were characterized by a higher Mcl-1 expression than the observed Bim level, which could be responsible for maintaining T cell reactivity, avoiding apoptosis activation after antigen encounter. Interestingly, these CD127-expressing cells from resolver patients down-regulated CD127 expression after specific *in vitro* challenge, linked to Mcl-1 down-regulation, without Bim expression up-regulation, acquiring an *in vitro* phenotype after expansion similar to the one observed in directly *ex vivo* cells from chronic patients. Therefore, these cells could be prone to apoptosis after re-stimulation, mimicking

the situation found in persistent infection. On the other hand, in cases with persistent high viral load, HCV-specific CTLs showed a $CD127^{low}$ phenotype that correlated with proliferation and γ-IFN production impairment. This phenotype linked with a directly *ex vivo* low Mcl-1 expression, similar to Bim levels that could allow apoptosis activation after Bim up-regulation. Actually, after specific *in vitro* TCR triggering, Bim up-regulation on $CD127^{low}$-expressing HCV-specific CTLs was observed. This finding was previously shown by our group [5], and it did not occur on $CD127^{high}$-expressing cells, as previously commented. In an experimental model, a similar observation was made showing that Bim was a critical mediator of apoptosis, induced by TCR re-stimulation, in the context of growth cytokine withdrawal [23]. Therefore, we could speculate that CD127 low expression on HCV-specific CTLs during persistent infection could favour Mcl-1 down-regulation on these cells, leading to apoptosis after antigen encounter, when Bim expression is up-regulated on HCV-specific CTLs, and Mcl-1 low level is unable to block Bim activity. In fact, the difference between Mcl-1 and Bim expression was significantly lower in $CD127^{low}$-expressing HCV-specific CTLs than in $CD127^{high}$ cells, and this correlated with T cell reactivity. These findings were antigen specific, because no difference in Mcl-1/Bim expression on total $CD8^+$ cells between the study groups was observed. Therefore, assuming that Mcl-1/Bim imbalance could be involved in the hyporeactivity of $CD127^{low}$-expressing HCV-specific CTLs through apoptosis activation, $CD127^{low}$ HCV-specific $CD8^+$ T cell reactivity rescue was attempted by interfering with caspase activity. Interestingly, $CD127^{low}$ HCV-specific $CD8^+$ T cell treatment with a pan-caspase inhibitor restored T cell reactivity to a similar range to the one observed in $CD127^{high}$ HCV-specific $CD8^+$ T cells from most HCV-resolved infection cases. These data suggested a link between apoptosis after TCR triggering and low CD127 expression on experienced HCV-specific CTLs during persistent infection that could be related to Mcl-1/Bim imbalance.

Bearing in mind these results, at least theoretically, therapeutic apoptosis blockade could help to restore the proliferation capacity of $CD127^{low}$/Mcl-1^{low}/Bim^{high} HCV-specific $CD8^+$ T cells after antigen encounter in chronic hepatitis C patients. This strategy could be a useful tool to try to improve the response to standard of care in chronic HCV patients. So far, the common calceneurin inhibitor cyclosporine-A has been shown to prevent T cell deletion through blocking Bim up-regulation [17]. Considering the *in vitro* HCV-specific CTL restoration, using the pancaspase inhibitor z-VAD-fmk, a drug as cyclosporine-A with a similar effect, but commonly used in some human diseases, could be employed in combination with anti-viral treatments to enhance the immune adaptive response. Another possible strategy to restore HCV-specific CTL reactivity during chronic infection could be the administration of IL-7, in order to increase the stimulation of the reduced number of IL-7R molecules on HCV-specific CTLs to modulate the balance between Bim and Mcl-1. In fact, in an animal model of cytotoxic T cell exhaustion, IL-7 treatment resulted in an increased T cell effector function and viral clearance [24].

In summary, this work suggests for the first time that CD127 phenotype modulates Bim and Mcl-1 expression on HCV-specific CTLs, and this affects T cell reactivity through apoptosis regulation. Specifically, during chronic infection, Mcl-1/Bim imbalance could be involved in $CD127^{low}$ HCV-specific CTL hyporeactivity, but it could be overcome by pancaspase inhibition.

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Bim-mediated apoptosis and PD-1/PD-L1 pathway impair reactivity of PD1$^+$/CD127$^-$ HCV-specific CD8$^+$ cells targeting the virus in chronic hepatitis C virus infection

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ABSTRACT

PD-1 molecule promotes anergy and IL-7 receptor (CD127) induces an anti-apoptotic effect on T cells. Correlation between PD-1/CD127 phenotype and hepatitis C virus (HCV)-specific CD8$^+$ cell reactivity in resolved infection (RI) after treatment and persistent HCV-infection (PI) was analysed. Directly *ex vivo*, PD-1 and CD127 expression on HCV-specific CD8$^+$ cells displayed a positive and negative correlation, respectively with viraemia. Proliferation after stimulation on PD-1$^-$/CD127$^+$ cells from RI cases was preserved, while it was impaired on PD-1$^+$/CD127$^-$ cells from PI patients. PD1$^+$/CD127$^+$ population was observed in PI, and these maintained expansion ability but they did not target the virus. Frequency of PI cases with HCV-specific CD8$^+$ cell proliferation increased after anti-PD-L1 and anti-apoptotic treatment. Bim expression on HCV-specific CD8$^+$ cells from PI patients was enhanced. In conclusion, during chronic HCV infection non-reactive HCV-specific CD8$^+$ cells targeting the virus are PD-1$^+$/CD127$^-$/Bim$^+$ and, blocking apoptosis and PD-1/PD-L1 pathway on them enhances *in vitro* reactivity.

1. Introduction

Hepatitis C virus (HCV) infection is often associated with persistent viraemia and progressive liver disease [1]. HCV-specific cytotoxic T lymphocytes (CTL) play a major role in spontaneous infection resolution. Nevertheless, during chronic infection these cells lack adequate effector functions and fail to control HCV [2].

Appropriate activation of experienced virus-specific CTL depends on the engagement between T cell receptor (TCR) and HLA-I/epitope complex [3] plus interaction between positive co-stimulatory molecules and their ligands [4]. These cells after developing their effector action, express negative co-stimulatory molecules, such as programmed death-1 (PD-1), to switch-off their activity [5]. Engagement of PD-1 and its ligand (PD-L1) delivers a negative signal avoiding proliferation and interleukin (IL)-2 production [6]. On the other hand, IL-7 receptor (CD127) plays an essential role in mature lymphocyte survival by counteracting the induction of apoptosis after antigen encounter through myeloid cell leukemia sequence-1 (Mcl-1) expression and Bcl2-interacting mediator (Bim) down-regulation, enhancing IL-2 secretion and life span [7].

HCV infection could modulate these two molecules to impair HCV-specific CTL reactivity through induction of either a virus-associated tolerogenic-like state or apoptosis. PD-1$^+$/CD127$^-$ phenotype associated with anergic features and apoptosis in chronic hepatitis C has been shown [8–10]. Nevertheless, HCV-specific CTL reactivity after antigen encounter in a proportion of HCV persistent infection cases are also maintained [11,12]. In this study the effect of PD-1/CD127 phenotype on HCV-specific CTL reactivity after antigen recognition according to viral control was analysed and the effect of modulating these molecules was tested.

2. Patients and methods

2.1. Patients

In this cross-sectional study ninety-four consecutive HLA-A2$^+$ subjects with genotype-1 HCV infection were recruited between January-2007 and August-2010 at Guadalajara University Hospital and "Fundación Jiménez Díaz" Hospital, Spain. These patients were divided into two groups according to their serum HCV-RNA and

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Table 1
Clinical features of patients enrolled in the study.

		Resolved infection	Persistent infection	*p*-value
N° of patients (n)		18	76	
SVR (n/%)		18 (100%)	–	
Relapser (n/%)		–	12 (16%)	–
Non responder (n/%)		–	10 (13%)	–
Naïve (n/%)		–	54 (71%)	–
HLA-A2 (%)		100	100	–
Age (years)		41 ± 7	47 ± 9.16	NS
Male sex (%)		70	68	NS
Evolution time (years)		24 ± 9	31 ± 12	NS
ALT (IU/L)		42.1 ± 19.2	92.8 ± 54.5	<0.001
Viral load (IU/mL)		<40	$2.12 \pm 3.7 \times 10^6$	<0.001
Genotype 1a/1b (%)		71/29	70/30	NS
Liver histology*				
	Lobular activity	1.82 ± 0.67†	1.97 ± 0.6	NS
	Portal activity	2.11 ± 0.56†	2.21 ± 0.6	NS
	Fibrosis	1.14 ± 0.45†	1.8 ± 0.7	NS

Data are presented as either percentage or mean ± standard deviation. NS: non-significant. SVR: sustained virologic response. *Liver histology is described according to Scheuer index. †Liver histology data just before anti-HCV treatment.

alanine transaminase (ALT) levels at the time of investigation; 76 patients with ALT > 40 IU/mL and HCV-RNA > 1000 IU/mL [hereafter indicated as persistent infection (PI)] (12 relapsers, 10 non-responders after anti-HCV treatment and 54 naïve) and 18 subjects with ALT < 40 IU/mL and HCV-RNA < 40 IU/mL, persistently maintained after developing sustained virologic response (SVR) subsequent to anti-HCV treatment [hereafter indicated as resolved infection (RI)] (Table 1). Other causes of chronic liver disease were excluded. 10 HLA-A2$^-$ HCV infected patients were taken as controls. In all recruited patients a serum and heparinised blood samples were taken. In treated patients, samples were taken 24 weeks after stopping treatment and in naïve patients at enrollment. All treated patients received treatment with pegylated interferon alfa-2b (1.5 ug/kg/week) and ribavirin (800–1400 mg/day) for 48 weeks following the standard stopping rules [13]. In nineteen PI patients and pre-treatment in all RI cases a liver biopsy was also performed. The study protocol was approved by the Regional Ethical Committee and patients gave written informed consent before enrolment.

2.2. Tissue typing

Screening for the HLA-A2 haplotype was performed by staining PBMC with FITC-conjugated anti-HLA-A2 mAb (Incstar, Stillwater, MN) and flow cytometric analysis.

2.3. Virological assessment

Anti-HCV was determined by Ortho HCV Version 3.0 ELISA Test (Ortho Diagnostic System, Raritan, NJ). HCV-RNA load was assessed by a quantitative real-time-PCR assay with a lower detection limit of 40 IU/mL (Cobas Amplicor HCV Monitor 2.0 Roche, Indianapolis, IN). HCV genotype was tested by InnoLiPPA HCV II assay (Innogentics Inc., Alpharetta, GA).

2.4. Synthetic peptides and pentamers

A2-restricted peptides corresponding to the genotype-1 $NS3_{1406-1415}$ region (KLVALGINAV), $NS3_{1073-1081}$ region (CINGVCWTV) and PE-conjugated HLA-A2 pentameric complexes (pentamer) loaded with the same two NS3 peptides and with cytomegalovirus (CMV) $pp65_{495-504}$ (NLVPMVATV) peptide were purchased from ProImmune (Oxford, UK).

2.5. CD8$^+$/pentamer$^+$ cell quantification

Peripheral blood (PBMC) and intrahepatic mononuclear cells (IHMC) were purified from heparinised blood samples and biopsies according to method described previously [14]. To quantify HCV-specific CD8$^+$ cells, $0.5–1 \times 10^6$ PBMC or a variable number of IHMC were incubated for 10 min at 37 °C with 1 μg of PE-labelled pentamers in RPMI 1640 plus 10% FCS. Cells were washed in PBS and then incubated at 4 °C for 20 min with saturating concentrations of directly conjugated anti-CD8-Pe-Cy5 (Cy) mAb, (Sigma–Aldrich Inc., St. Louis, MO). Subsequently, stained cells were analysed after washing on a Becton Dickinson FACS® using CELLQuest™ software. The cut-off for pentamer assay was determined by staining intrahepatic and peripheral blood mononuclear cells from 10 HLA-A2$^-$ PI patients. Using the mean plus 2 standard deviations as the cut-off point, the lowest percentage at which pentamer-binding CD8$^+$ cells could be detected as a separate cell population was 0.018% out of total CD8$^+$ cells.

2.6. PD-1/CD127 phenotypic analysis on CD8$^+$/pentamer$^+$ cell

In patients with proven pentamer$^+$ cells, phenotypic analysis was performed. PBMC and IHMC were triple stained with PE-pentamers plus anti-CD8-Cy mAb as above and either FITC-conjugated anti-CD127 (Pharmingen BD, San Jose, CA) or anti-PD-1 (eBiosicience Inc., San Diego, CA) mAbs. Subsequently, stained cells were analysed by flow-cytometry. Isotype-matched control mAbs were used to set the markers for the different stainings. Different phenotypes were quantified on pentamer$^+$ cells as the mean fluorescence intensity (MFI) for each staining, and compared between the study groups. Phenotypes were performed directly *ex vivo* in circulating and liver-infiltrating pentamer$^+$ cells and after specific *in vitro* challenge in the peripheral compartment. Depending on the MFI, PD-1 and CD127 phenotypes were marked as: low (−), intermediate (+) and high (++).

2.7. Production of T cell lines

PBMC were re-suspended at a concentration of 1×10^6/ml in RPMI 1640 plus 10% FCS. Cells were stimulated with 1 μM of one of these two NS3 specific peptides, $NS3_{1406-1415}$ and $NS3_{1073-1081}$, in a 96-well plate. Recombinant IL-2 (25 IU/ml) was added on day 2 of culture, and cells were analysed after a total of 10 days of culture. After specific *in vitro* expansion, pentamer$^+$ cell phenotype was again analysed as above. T cells from selected RI and PI patients were also cultured in presence or absence of either anti-PD-L1 blocking mAb (2 μg/mL) (Santa Cruz Biotechnology, Santa Cruz, CA) or the pancaspase inhibitor zVAD-fmk (50 μM) (BD Bioscience, San Jose, CA), using in both cases β-galactosidase (2 μg/mL) (Santa Cruz Biotechnology, Santa Cruz, CA) as control. Expansion was considered positive when a pentamer-binding CD8$^+$ cell population bigger than base line and higher than 0.04% out of total CD8$^+$ cells was observed after specific stimulation.

2.8. HCV-epitope sequencing

In PI patients with directly *ex vivo* $NS3_{1406-1415}$ or $NS3_{1073-1081}$-pentamer binding CD8$^+$ cells, epitope was sequenced in order to test whether PD-1/CD127 phenotype could be affected by HCV sequence variation. Patients' plasma were collected by centrifugation in plasma preparation tubes and frozen immediately at −80 °C. Total HCV-RNA was isolated using QIAamp Viral RNA Kit (Qiagen, Germany) and, reverse transcribed. Subsequently, nested-PCR

Table 2
HCV primers for amplification and sequencing.

		Sense	Antisense
NS31406–1415 (KLVALGINAV)	First PCR	(5′-ACGTACTCCACCTACGGCAA-3′)	(5′-AAGGTAGGGTCAAGGCTGAA-3′)
	Second PCR	(5′-CATCCCAACATCGAGGAGGT-3′)	(5′-TTGCAGTCTATCACCGAGTC-3′)
NS31073–1081 (CINGVCWTV)	First PCR	(5′-GGCYTGCCCGTCTCYGCCCG -3′)	(5′-CGGCGCACSGGAATGACATCG -3′)
	Second PCR	(5′-CGGCSTACKCCCARCAGACGMGAGGCC-3′)	(5′-CCTCGTGACCARGTAAAGGTCC-3′)

PCR conditions were as follows: 1 cycle (95 °C 5 min); 35 cycles (95 °C 45 s, 42 °C 45 s, 72 °C 45 s) and 1 cycle (72 °C 10 min).

amplification was carried out, using the NS3 primers described in Table 2. A single band of the expected size was obtained. Final PCR products were purified using GeneClean kit (Bio101, La Jolla, CA) and, subsequently sequenced in an ABI PRISM® 377 DNA sequencer (Applied Biosystems Inc., Foster City, CA).

2.9. Intracellular Bim staining

Directly *ex vivo* and after specific *in vitro* challenge in presence of zVAD-fmk, HCV-specific $CD8^+$ T cells from selected RI and PI cases were surface stained with PE-pentamers and CD8-Cy mAb as above. After a further wash, cells were subjected to intracellular staining using Cytofix-Cytoperm (BD Bioscience, San Jose, CA) to permeabilise and fix cells according to the manufacturer's instructions, followed by staining with Bim unconjugated Ab (Cell Signaling Technology, Beverly, MA) plus goat anti-rabbit IgG2 Alexa 488 (Invitrogen, Carlsbad, CA) and its isotype-matched control (Pharmingen BD, San Jose, CA). Finally, cells were washed twice and analysed by flow cytometry and expressed as MFI Bim-Alexa 488 on $CD8^+$/pentamer$^+$ cells.

2.10. Statistical analysis

Categorical data are presented as either percentage or 95% confidence interval for percentage (CIP) whereas continuous variables are summarised as median plus interquartile range (IQR) or mean ± standard deviation. Wilcoxon, Mann–Whitney-*U* and Pearson tests were employed where appropriate. Significance was established at $p < 0.05$. Statistical analysis of frequency, phenotype and proliferation of pentamer$^+$ cells was performed pooling the results for the different epitopes tested in a single variable. For correlation tests, HCV viral load and PD-1 MFI were log transformed in order to make these variables follow a normal distribution.

3. Results

3.1. PD-1/CD127 expression on intrahepatic and circulating $CD8^+$/pentamer$^+$ cells

Mononuclear cells from PI patients' liver biopsies and peripheral blood samples were studied. A portion of these cells were directly *ex vivo* double stained with anti-CD8-Cy mAbs and with PE-pentamers to test the presence of HCV-specific $CD8^+$ cells against the two NS3 immunodominant epitopes studied. In 74% and 89% of liver biopsies from PI patients, intrahepatic pentamer$^+$ cells against $NS3_{1406–1415}$ and $NS3_{1073–1081}$ epitopes, respectively were observed with a mean global percentage of 78% (22 out of 28 tests; 95% CIP: 60–91%), while only in 14–28% of PI patients, according to the pentamer tested, there were cells in peripheral blood also, with a mean percentage of 23% (23 out of 115 tests; 95% CIP: 12–25%) (Table 3). Therefore, an intrahepatic sequestration of pentamer-binding $CD8^+$ cells was shown ($p < 0.001$; Table 3) with a median intrahepatic frequency of 0.35% (IQR: 3.7) pentamer$^+$ cells out of total liver-infiltrating $CD8^+$ cells, while median frequency of peripheral pentamer-binding $CD8^+$ cells was 0.046% out of total $CD8^+$ cells (IQR 0.07), in the cases with detectable cells. In patients with RI, the frequency of cases with detectable pentamer$^+$ cells in peripheral blood was also very low, between 20% and 22%, depending on the NS3 epitope tested, with a mean percentage of 21% (7 out of 33; 95% CIP: 7–35%), which was similar to the one observed in PI (p = NS; Table 3). The median frequency of peripheral pentamer-binding $CD8^+$ cells in RI patients with detectable cells was also low (0.056%; IQR: 0.05) and similar to the frequency found in PI cases (p = NS; Table 3). In patients with directly *ex vivo* detectable pentamer$^+$ cells, mononuclear cells were triple stained with PE-pentamers and anti-CD8-Cy mAb plus either anti-PD1-FITC or anti-CD127-FITC mAbs. The PD-1 FITC MFI on intrahepatic pentamer-binding cells was higher (++) (105; IQR: 94) than the MFI observed in peripheral blood (+) (34; IQR: 25) for PI cases ($p = 0.04$; Fig. 1A and B). PD-1 FITC MFI on peripheral HCV-pentamer$^+$ cells from RI patients was much lower (−) (8; IQR: 5) than the MFI observed in PI ($p < 0.001$; Fig. 1A and B) and similar to the intensity observed for peripheral CMV-pentamer$^+$ cells from PI patients. On the other hand, CD127-FITC MFI on intrahepatic pentamer$^+$ cells was lower (−) (5.1; IQR: 1.2) than in the peripheral compartment (+) (13; IQR: 9) in PI ($p = 0.03$; Fig. 1A and B), while CD127 expression on peripheral HCV-pentamer$^+$ cells from RI patients was much higher (++) (32; IQR 15) than in PI ($p < 0.001$; Fig. 1A and B) and similar to the expression on peripheral CMV-pentamer$^+$ cells from PI subjects. In the group of PI patients, these data translated into a significant positive and negative correlation between HCV viral load and PD-1 ($r = 0.443$; $p = 0.03$) and CD127 expression ($r = -0.456$; $p = 0.038$) on peripheral pentamer$^+$ cells respectively (Fig. 2).

3.2. Proliferation ability of $CD8^+$/pentamer$^+$ cells after antigen encounter

The capacity for circulating pentamer$^+$/$CD8^+$ cells to expand after exposure to viral antigens according to viral control in enrolled patients was investigated. A different clonal expansion potential between PI and RI subjects was observed. Specifically, in 22% (5 out of 22) and 39% (21 out of 53) of PI, and in 82% (9 out of 11) and 72% (13 out of 18) of RI cases an expansion after $NS3_{1073–1081}$ and $NS3_{1406–1415}$ stimulation, respectively was shown ($p < 0.001$ and $p = 0.018$, respectively; Fig. 3A). The proportion of experiments with expansion, taking together the results for both epitopes, was higher in RI (22 out of 29; 76%: 95% CIP [59–92%]) than in PI (26 out of 75; 34%: 95% CIP [23–45%]) ($p < 0.001$; Fig. 3A and B). PD-1/CD127 phenotype was different between PI and RI patients after standard proliferation. PD-1 FITC MFI on pentamer$^+$ cells from PI cases was significantly higher (+) (32.3; IQR 32.3) than in RI subjects (−) (8.3; IQR 8.1) ($p = 0.001$; Fig. 3C). Interestingly, CD127 expression on pentamer$^+$ cells from RI patients was low (8.1; IQR 6.4) (−) after *in vitro* challenge, as would be expected for effector cells after antigen encounter ($p = 0.03$; Fig. 3C). Nevertheless, pentamer$^+$ cells from PI cases with expansion ability without any extra-treatment displayed an intermediate CD127-FITC MFI (+) (17.6; IQR 17.25) after proliferation which was higher than in expanded cells from RI subjects (−) ($p = 0.022$; Fig. 3C).

Table 3
Number of patients tested directly *ex vivo* for the presence of pentamer$^+$ cells against two different NS3-HCV immunodominant epitopes, and frequency of these cells in patients with detectable pentamer$^+$ cells.

			All epitopes	NS31406–1415	NS31073–1081	*p*-value
PI	IH	N^0 of cases tested (n)	28	19	9	
		Cases with detectable pentamer + cells (n; %)	22 (78%)	14 (74%)	8 (89%)	<0.001*
		Frequency of Pent + cells out of total CD8 + cells (%)†	0.36 (IQR 3.7)	0.21 (IQR 3.9)	2.1 (IQR 3.8)	
	PB	N^0 of cases tested (n)	115	76	39	
		Cases with detectable pentamer + cells (n;%)	23(20%)	11(14%)	11(28%)	
		Frequency of Pent + cells out of total CD8 + cells (%)†	0.046 (IQR 0.07)	0.048 (IQR 0.04)	0.07 (IQR 0.09)	NS*
RI	PB	N^0 of cases tested (n)	33	18	15	
		Cases with detectable pentamer + cells (n;%)	7(22%)	4(22%)	3(20%)	
		Frequency of Pent + cells out of total CD8 + cells (%)†	0.056 (IQR 0.05)	0.064 (IQR 0.08)	0.054 (IQR NA)	

PI: persistent HCV infection. RI: resolved HCV infection. IH: intrahepatic. PB: peripheral blood. *Pearson Chi-square test. NA: non applicable due to sample size. Pent: pentamer. †Median frequency of pentamer$^+$ cells in patients with detectable pentamer$^+$ cells. The lower limit of detection with pentamer staining technique was 0.018% pentamer$^+$ cells out of total CD8$^+$ cells.

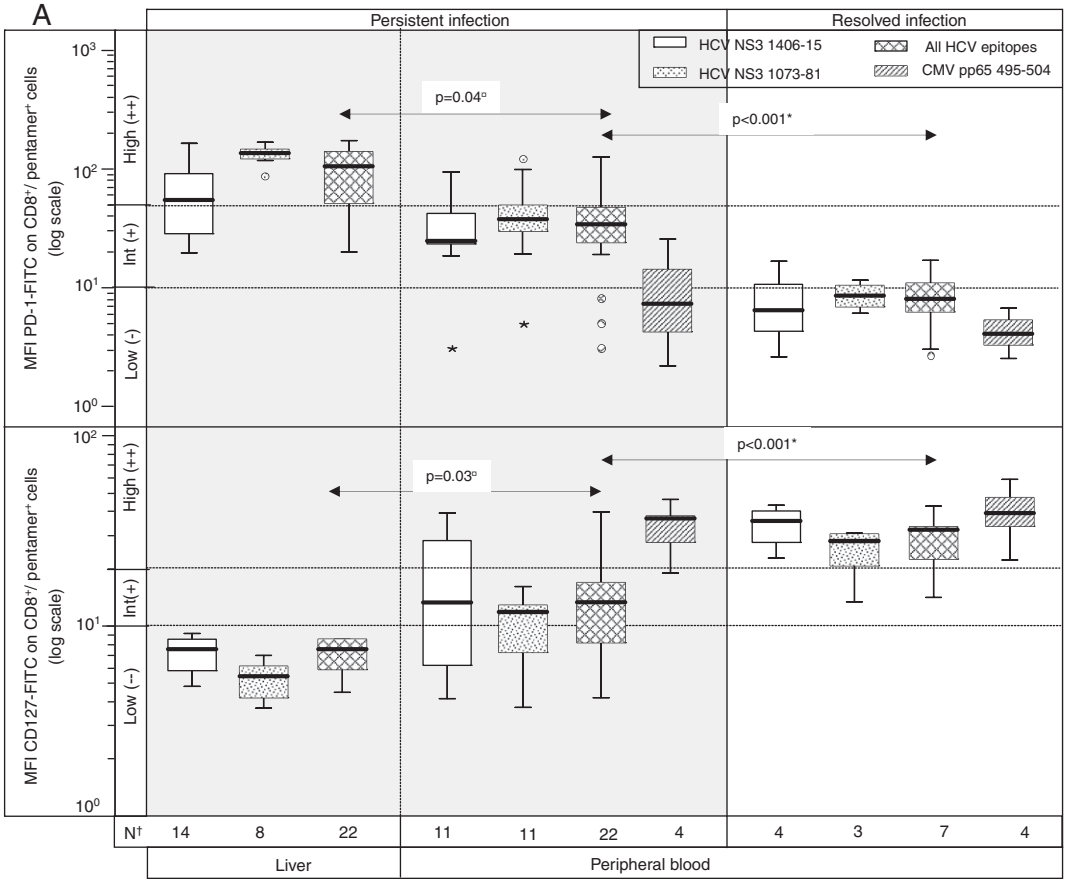

Fig. 1. Directly *ex vivo* analysis of PD-1/CD127 phenotype on pentamer-binding CD8$^+$ cells in HCV infection according to viral control. (A) Box-plots summarising the PD-1/CD127 MFI on CD8$^+$/Pentamer$^+$ cells against the different epitopes tested in resolved and persistent HCV patients. Int: intermediate. NS: non significant. O: outlier. *: extreme value. *Mann–Whitney *U* test. ºWilcoxon test. †Number of cases studied for every epitope. (B) FACS® dot-plots of peripheral and intrahepatic lymphocytes stained with CD8-Cy mAb, Pentamer-PE and either PD-1$^-$FITC or CD127-FITC mAbs, and FACS® histograms for PD1-FITC and CD127-FITC mAbs intensity of fluorescence gated on CD8$^+$/Pentamer$^+$ cells. The plots and histograms are representative from one patient with persistent infection and one subject with resolved infection. Upper left figures in each dot-plot represent the percentage of positive Pentamer$^+$ cells out of total CD8$^+$ cells. (−) Low, (+) intermediate and (++) high fluorescence.

3.3. Pentamer-binding CD8$^+$ cell populations according to CD127 expression in PI

In those cases with directly *ex vivo* detectable pentamer-binding CD8$^+$ cells, PD-1/CD127 phenotype and expansion ability were correlated. The analysis of all the performed experiments together showed that independently of HCV control and PD-1 level, CD127 expression correlated positively with pentamer-binding cell expansion capacity. In fact, 83% of CD127-expressing pentamer$^+$ cells expanded while only 16% of CD127$^-$ did expand (Fig. 4A and B; $p = 0.001$). PD-1 expression also associated with an impaired expansion ability but only in the CD127$^-$ cell subset (Fig. 4A and B; $p = 0.034$). After this preliminary study, the experiments were analysed according to viral control. All RI cases with detectable HCV-pentamer binding CD8$^+$ cells displayed a PD-1$^-$/CD127$^+$ phenotype (Fig. 4A), while in PI, two different CD127-expressing pop-

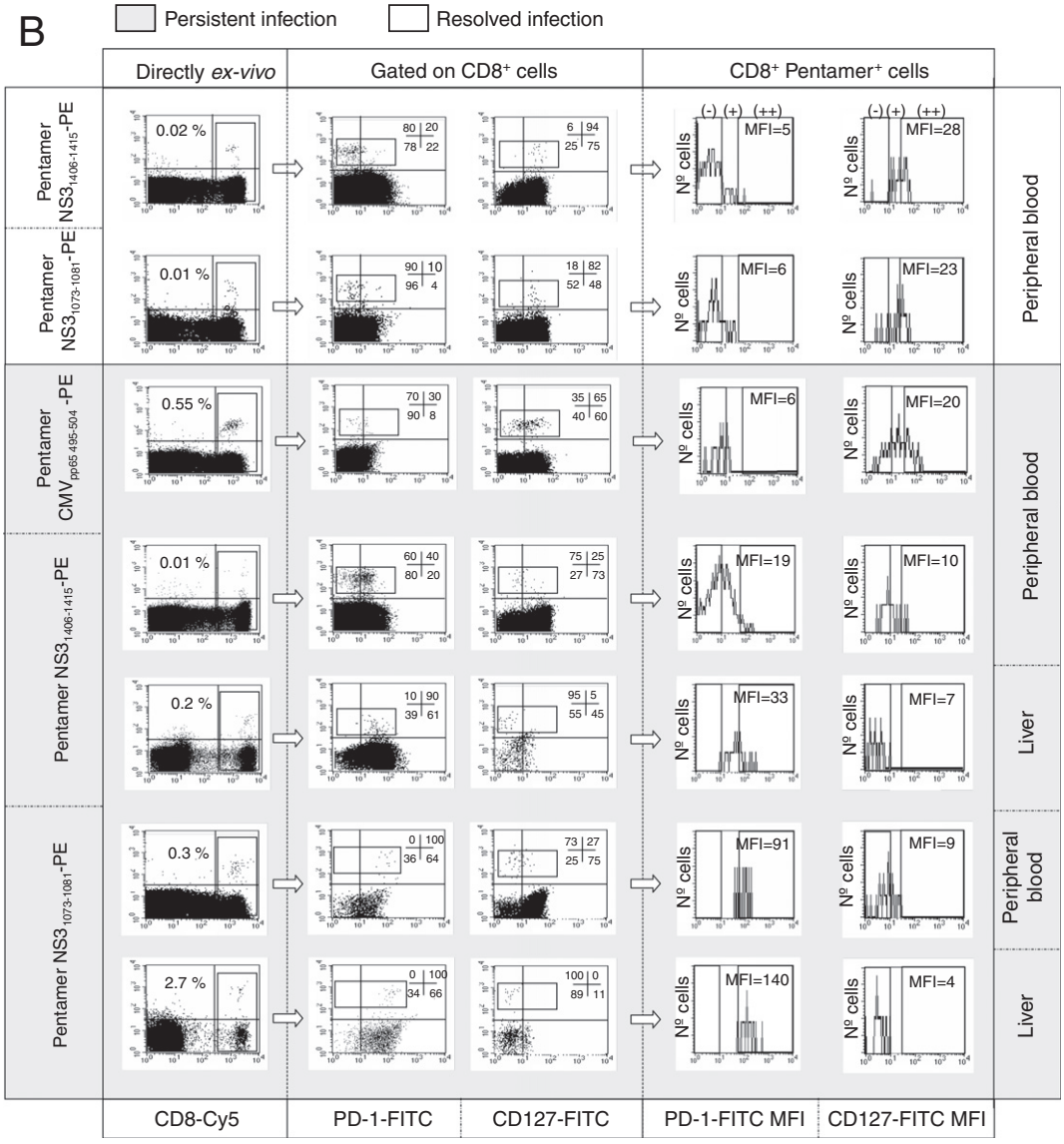

Fig. 1 (*continued*)

ulations with different expansion potential were observed (Fig. 4A). In PI, pentamer$^+$/CD8$^+$ cells maintaining expansion ability after antigen encounter expressed a higher CD127 expression (MFI 23.03; IQR 16) than cells without proliferation capacity (MFI 6.04; IQR 3.9) (Fig. 4C and D; $p = 0.045$). Nevertheless, independently of the pentamer-binding cell proliferative potential, PD-1 expression was high and similar (Fig. 4C and D; p = NS). In eight out of these 13 PI patients with directly *ex vivo* pentamer-binding CD8$^+$ cells, we were able to sequence HCV-epitopes. The presence of epitope mutations at TCR interaction sites was associated with a higher CD127 expression (Fig. 5A and B; $p = 0.048$), lower PD-1 expression (Fig. 5A and B; $p = 0.034$), and a different proliferation ability, although the last one did not reach statistical significance (Fig. 5C; $p = 0.112$), probably due to the small sample size.

3.4. Effect of apoptosis and PD-1/PD-L1 pathway blocking on pentamer-binding CD8$^+$ cell reactivity

The effect of PD-1/PD-L1 interaction blocking on T cell proliferation was tested by paired incubation with either anti-PD-L1 mAb or β-galactosidase as control, during T cell HCV-specific *in vitro* challenge in selected RI and PI patients. In PI patients, anti-PD-L1 mAb treatment increased significantly from 29% (8 out of 27; 95% CIP [11–48%]) up to 52% (14 out of 27; 95% CIP [31–72%]) the percentage of experiments with specific CTL proliferation after $NS3_{1073-1081}$ or $NS3_{1406-1415}$ stimulation ($p = 0.023$; Fig. 6A and B). However, this treatment did not increase the frequency of cases with expansion on RI patients (13 out of 18: 72%; 95% CIP [49–95%]) (p = NS; Fig. 6A and B), probably due to the low PD-1 expression on these cases. Finally, we tested the effect of blocking apoptosis by pancaspase inhibitor zVAD-fmk on pentamer$^+$ cell proliferation in PI and RI cases. This treatment was performed to counteract the presumable pro-apoptotic effect of IL-7 deprivation on CD127$^-$ pentamer$^+$ cells from PI cases. PBMC were *in vitro* challenged with either $NS3_{1406-1415}$ or $NS3_{1073-1081}$ in presence of zVAD-fmk or β-galactosidase as control. Blocking apoptosis increased the frequency of experiments with positive proliferation in PI from 23% (3 out of 13; 95% CIP [0–49%]) up to 54% (7 out of 13; 95% CIP [22–85%]) ($p = 0.018$; Fig. 6C and D). Nevertheless, anti-apoptotic treatment did not affect the proliferation ability of pentamer-binding CD8$^+$ cells after specific *in vitro* challenge in RI cases (8 out of 11: 72%; 95%CIP [41–100%]) (p = NS; Fig. 6C and

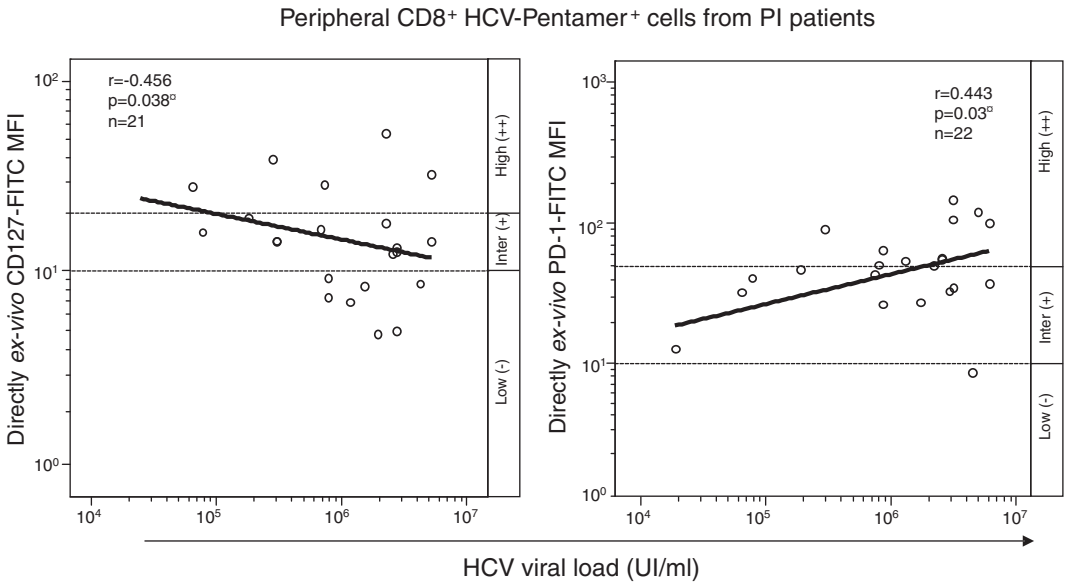

Fig. 2. Correlation between PD-1/CD127 phenotype and viral load in PI patients. Scatter-plot showing the correlation between PD-1/CD127 expression on pentamer binding CD8$^+$ cells and viral load. ¤Pearson's correlation coeficient. Inter: intermediate. MFI: mean fluorescence intensity. PI: persitent infection.

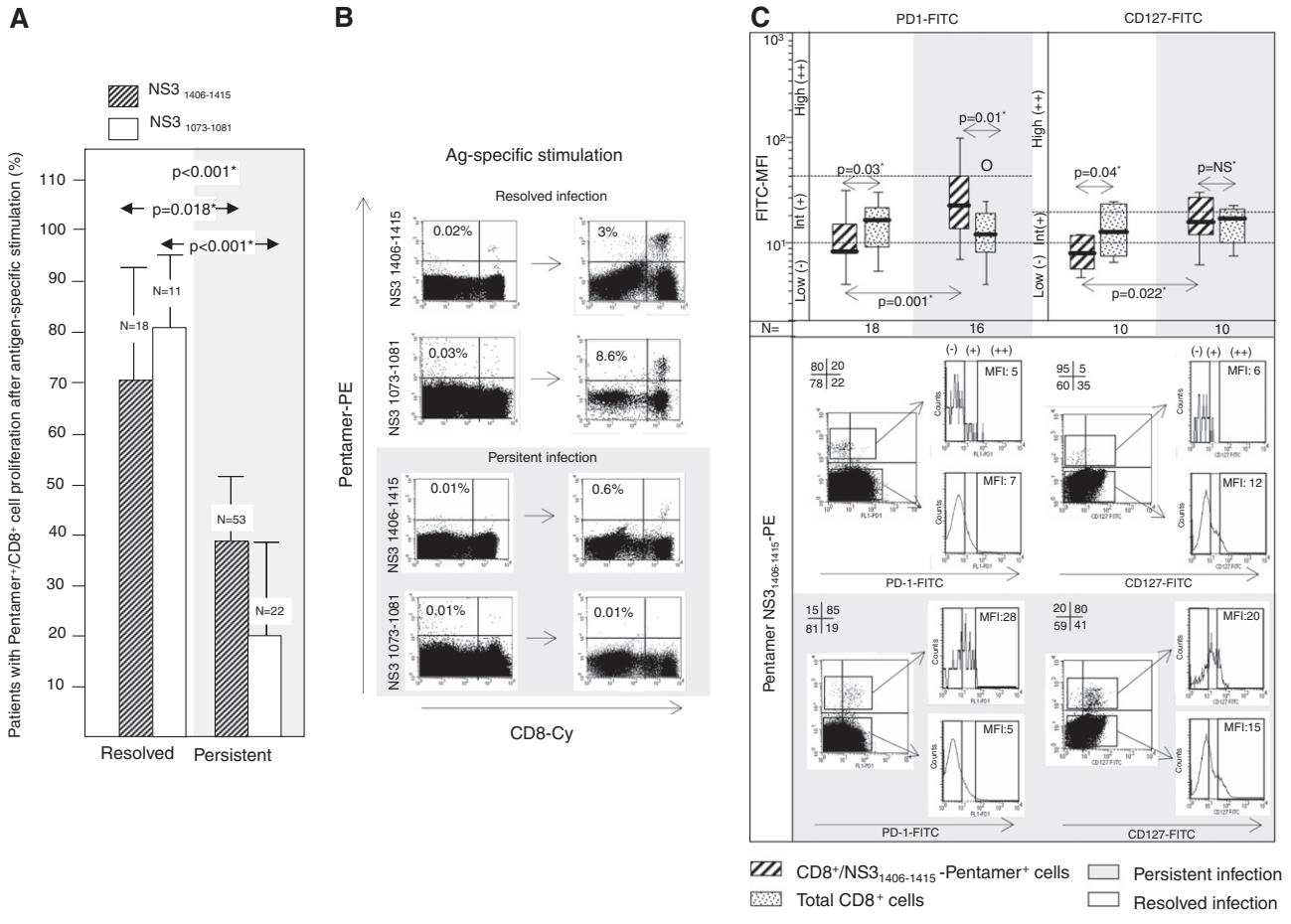

Fig. 3. Pentamer-binding CD8$^+$ cells expansion ability after specific *in vitro* challenge according to viral control. (A). Bar-plots showing the frequency of experiments with pentamer-binding cell expansion after specific *in vitro* challenge in PI and RI. Whiskers represent 2× standard error of the percentage. ∗Mann–Whitney-*U* test. (B). FACS® dot-plots of peripheral mononuclear cells stained with PE-labelled Pentamers and CD8-Cy mAb directly *ex vivo* and after specific stimulation from representative PI and RI patients. Upper left figures in each dot-plot represent the percentage of positive Pentamer$^+$ cells out of total CD8$^+$ cells. (C) Box-plots showing the PD-1 and CD127 FITC-MFI on pentamer binding CD8+ cells and total CD8$^+$ cells, as internal control, after specific *in vitro* challenge in PI and RI. Representative FACS® fluorescence histograms of PD-1 and CD127 FITC on total CD8$^+$ and pentamer-binding CD8$^+$ cells from one RI and one PI patient are shown. ∗Mann–Whitney *U* test. Int: intermediate.

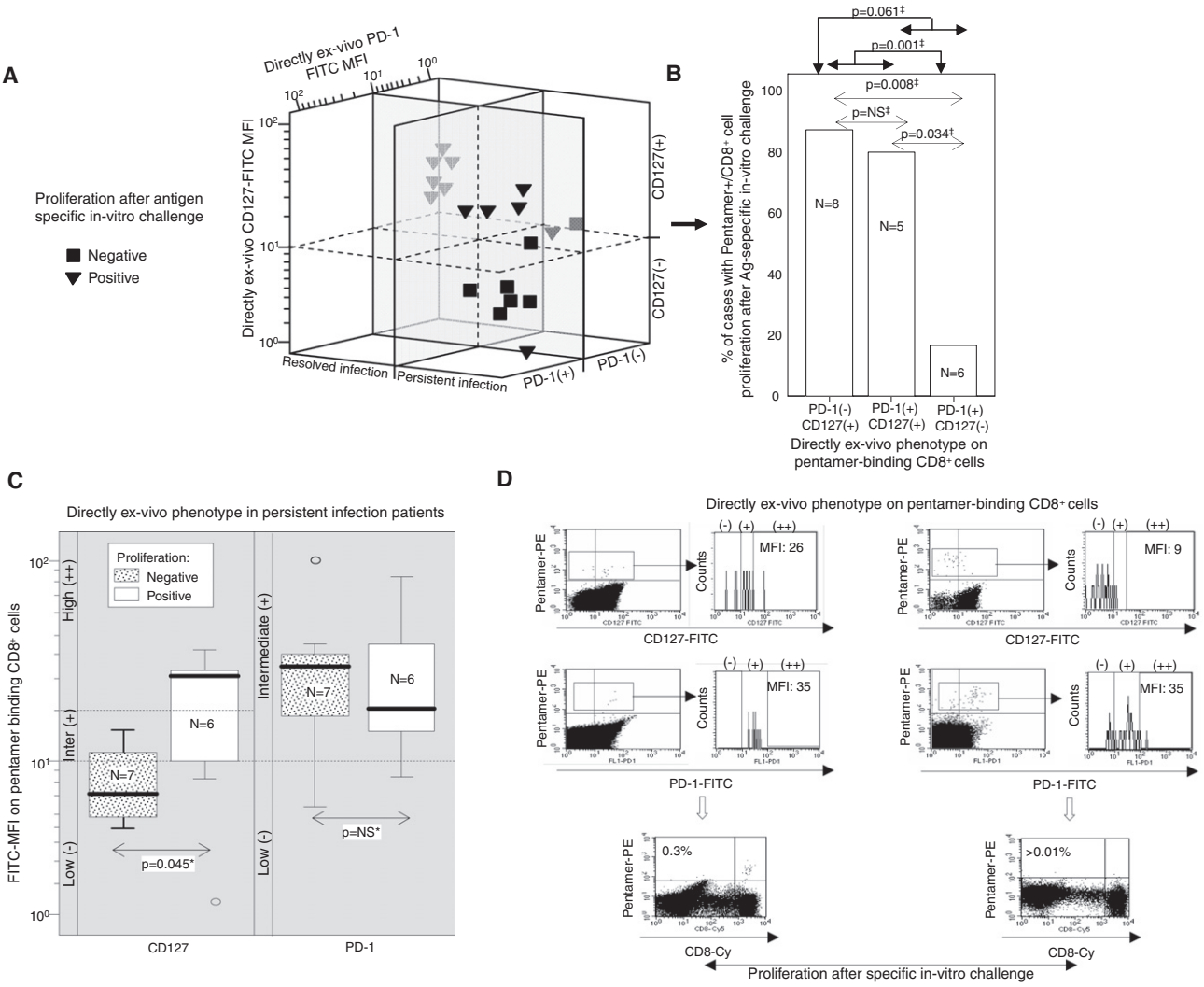

Fig. 4. Association between directly *ex vivo* PD-1/CD127 phenotype and pentamer-binding cell reactivity after antigen encounter. (A) Three-dimensional scatter plot showing the PD-1 and CD127 FITC MFI on peripheral pentamer-binding cells according to viral control and expansion ability. (B) Bar-plots showing the percentage of experiments with proliferation according to PD-1/CD127 phenotype on $CD8^+$/$Pentamer^+$ cells. The three different PD-1/CD127 subsets are compared between them (small arrows) and also PD-1 and CD127 phenotypes are compared independently (big arrows). ‡Man-Whitney *U* test. (C) Box-plots showing the PD-1 and CD127 FITC-MFI on pentamer-binding cells according to proliferation ability in patients with PI. ∗Mann–Whitney *U* test. O: outlier. Inter: intermediate. NS: non significant. (D) Representative FACS® dot-plots and fluorescence histograms of peripheral pentamer-binding cells stained with PD-1- FITC and CD127-FITC mAbs from two PI patients with different expansion ability after specific *in vitro* challenge. Upper left figures in each dot-plot represent the percentage of positive $pentamer^+$ cells out of total $CD8^+$ cells. (−) Low, (+) intermediate and (++) high fluorescence.

D). Directly *ex vivo* and after specific *in vitro* expansion in presence of zVAD-fmk treatment, Bim expression on pentamer-binding $CD8^+$ cells from PI and RI patients was also tested. Directly *ex vivo*, Bim expression was higher on PI patients (MFI 166; IQR: 151) than in RI cases (MFI 118; IQR: 93), although this difference was not statistically significant, probably due to the sample size. However, pentamer-binding $CD8^+$ cells from PI cases after specific *in vitro* expansion in presence of zVAD-fmk displayed a significantly higher Bim expression (MFI 221; IQR: 331) than RI patients (MFI 77; IQR: 32) (Fig. 6D and E; $p < 0.001$).

4. Discussion

In persistent HCV infection, specific cytotoxic response is weak and unable to clear the virus [2]. Sustained immunological viral pressure in chronic non-cytopathic viral infection could induce an anergic phase and a subsequent deletion on specific CTL [15,16]. In this process, the expression of the negative co-stimulatory molecule PD-1 [17] and down-regulation of the anti-apoptotic IL-7 receptor [18] could take part. In this study the effect of these two molecules on HCV-specific CTL reactivity according to viral control was analysed (See Table 4).

In the majority of PI patients studied in this work, intrahepatic HCV-specific $CD8^+$ cells were demonstrated and circulating cells were also detectable in a few patients. These data suggested that there did not occur a specific CTL deletion in most chronic patients despite of the intrahepatic apoptotic process previously described [10], but that these cells were sequestered in the liver and below the detection threshold in the peripheral compartment. These cells were characterised by a PD-1/CD127 phenotype associated with anergy and apoptosis [9,10], which could conduct them to die in the liver during chronic infection. Therefore, there probably is a continuous recruitment of newly generated HCV-specific $CD8^+$ cells to maintain the intrahepatic population of specific CTL as it has recently been described in LCMV infection [19]. As a result, the fact that these cells in chronic infection are not completely deleted but dysfunctional is important information, because it could

A

ID	Genotype	Epitope	Sequence	PD-1 MFI	CD127 MFI	Exp
144	1a	$NS3_{1073-1081}$	Wild type	38	4	Neg
270	1b	$NS3_{1073-1081}$	Wild type	31	7	Neg
42	1a	$NS3_{1406-1415}$	Wild type	39	ND	Neg
184	1b	$NS3_{1073-1081}$	Wild type	31	8	Pos
292	1b	$NS3_{1073-1081}$	Wild type	ND	7	Pos
270	1b	$NS3_{1406-1415}$	L1410M, I1412V	19	12	Pos
300	1a	$NS3_{1406-1415}$	L1410M, I1412V	26	27	Pos
140	1b	$NS3_{1406-1415}$	A1409G, I1412V	12	28	Pos

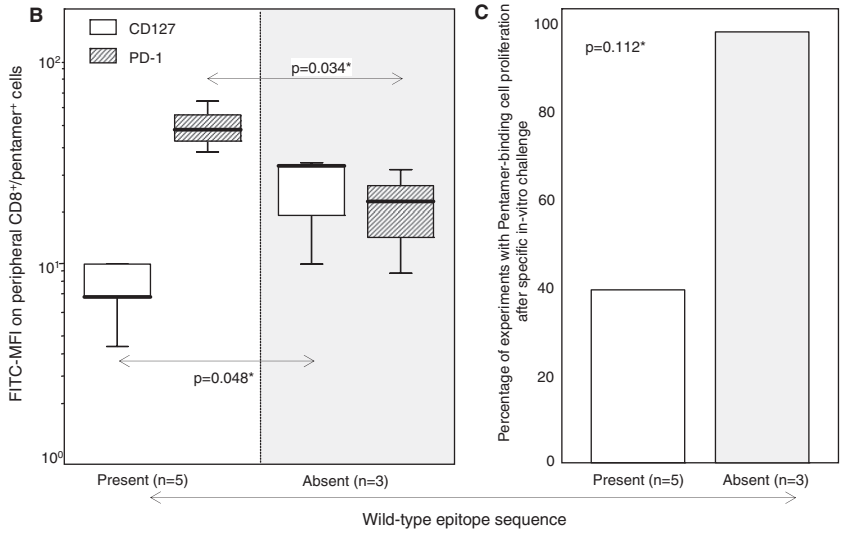

Fig. 5. PD-1/CD127 phenotype on pentamer-binding cells according to epitope sequence. (A) Table showing the PD-1 and CD127 FITC-MFI, the HCV-epitope sequence and the proliferation ability after antigen encounter in eight PI patients with directly *ex vivo* detectable pentamer$^+$ cells. Exp: expansion. (B) Box-plots displaying the PD-1 and CD127 FITC-MFI on peripheral pentamer-binding cells according to the presence or absence of wild-type HCV-epitope. ∗Mann–Whitney *U* test. (C) Bar-plot showing the percentage of experiments with proliferation of pentamer-binding CD8$^+$ cells after specific *in vitro* challenge according to the HCV-epitope sequence. ∗Mann–Whitney *U* test.

encourage us to search for mechanisms to restore their function in order to clear the HCV infection.

To asses the role of PD-1 and CD127 molecules on HCV-specific CTL reactivity, we analysed deeper the effect of these molecules on peripheral T cell expansion ability. Interestingly, in chronic infection PD-1/CD127 phenotype on circulating pentamer$^+$ cells correlated with level of viraemia. HCV-specific CTL submitted to high viral load displayed an anergic PD-1$^+$/CD127$^-$ phenotype, probably due to persistent ineffective CTL triggering as it has been shown in other viral infections [16]. The higher the viral load in chronic patients, the more intense the PD-1$^+$/CD127$^-$ phenotype is on HCV-specific CTL. The correlation between PD-1 expression and HCV viral load has been described previously [20], but this is the first report showing a negative correlation between CD127 expression and HCV viraemia. Moreover, a gradient for PD-1/CD127 expression between peripheral and intrahepatic compartment in PI was also observed. Consequently, the level of persistent HCV antigenemia was able to regulate the expression of these two molecules on HCV-specific CD8$^+$ cells, being this modulation more intense in the HCV replication site where the antigenemia is higher [21]. Clearly this could be a HCV evolutionary mechanism to escape from immune control.

In the complete cohort of patients enrolled in the study, high specific-CTL reactivity in RI after anti-HCV treatment was observed, suggesting that these cells could be important to obtain a SVR. Whether it is necessary to restore the HCV-specific CTL response to obtain a SVR after treatment is not known yet but our cross-sectional data, and some previous work suggest that this could be required [22]. On the other hand, in chronic infection an impaired reactivity of HCV-specific CD8$^+$ cells after antigen encounter was demonstrated, and this was related with the PD-1$^-$/CD127$^+$ phenotype. Nevertheless, some PI cases kept spontaneous HCV-specific CD8$^+$ cell proliferation after specific *in vitro* challenge. In order to test whether these cells displayed a different PD-1/CD127 phenotype, in patients with directly *ex vivo* detectable HCV-specific CD8$^+$ cells, PD-1/CD127 phenotype and expansion ability were correlated. Directly *ex vivo* reactive HCV-specific CTL during PI displayed an unexpected high CD127 and low PD-1 expression. These cells may be able to escape sensitization to apoptosis after antigen encounter by maximizing rescue signals from IL-7 such as the anti-apoptotic protein Mcl-1 [23]. This phenotype may be a reflection of the recently primed status of the detectable HCV-specific CD8$^+$ T cells; such continuous recruitment of newly generated T cells to the ongoing response has been previously described in some viral persistent infections [19]. Nevertheless, our HCV-epitope sequencing data suggest that this fact could also be due to absence of immunological pressure, produced by viral variation on the targeted epitope, in agreement with recent previous works [12,24,25]. In this last case, PD-1/CD127 phenotype in PI could be a tool to discover CTL escape mutations. Interestingly, PD-1 expression was slightly increased in CD127$^+$ PI patients with respect to the level that should be expected due to the absence of viral pressure. This detail could be due to a non-TCR dependent PD-1 up-regulation induced by HCV infection, as it has been previously suggested by the effect of HCV-core protein [26]. These reactive cells from PI patients, non-targeting the virus, maintain a higher CD127 expression after expansion than cells from RI patients, probably suggesting that CD127$^+$ cells from RI and PI patients belong to different T cell subsets with distinct degree of differentiation. The status of CD8$^+$ T cells is influenced by the history of antigen stimulation [27,28], therefore in the case of HCV escape mutations the long absence of antigen stimulation could provoke a naïve-early phenotype in peripheral HCV-specific CTL while in RI, these cells would present and early-intermediate phenotype ready to transform into effector-memory cells after antigen encounter. On the other hand, non-reactive HCV-specific CTL tar-

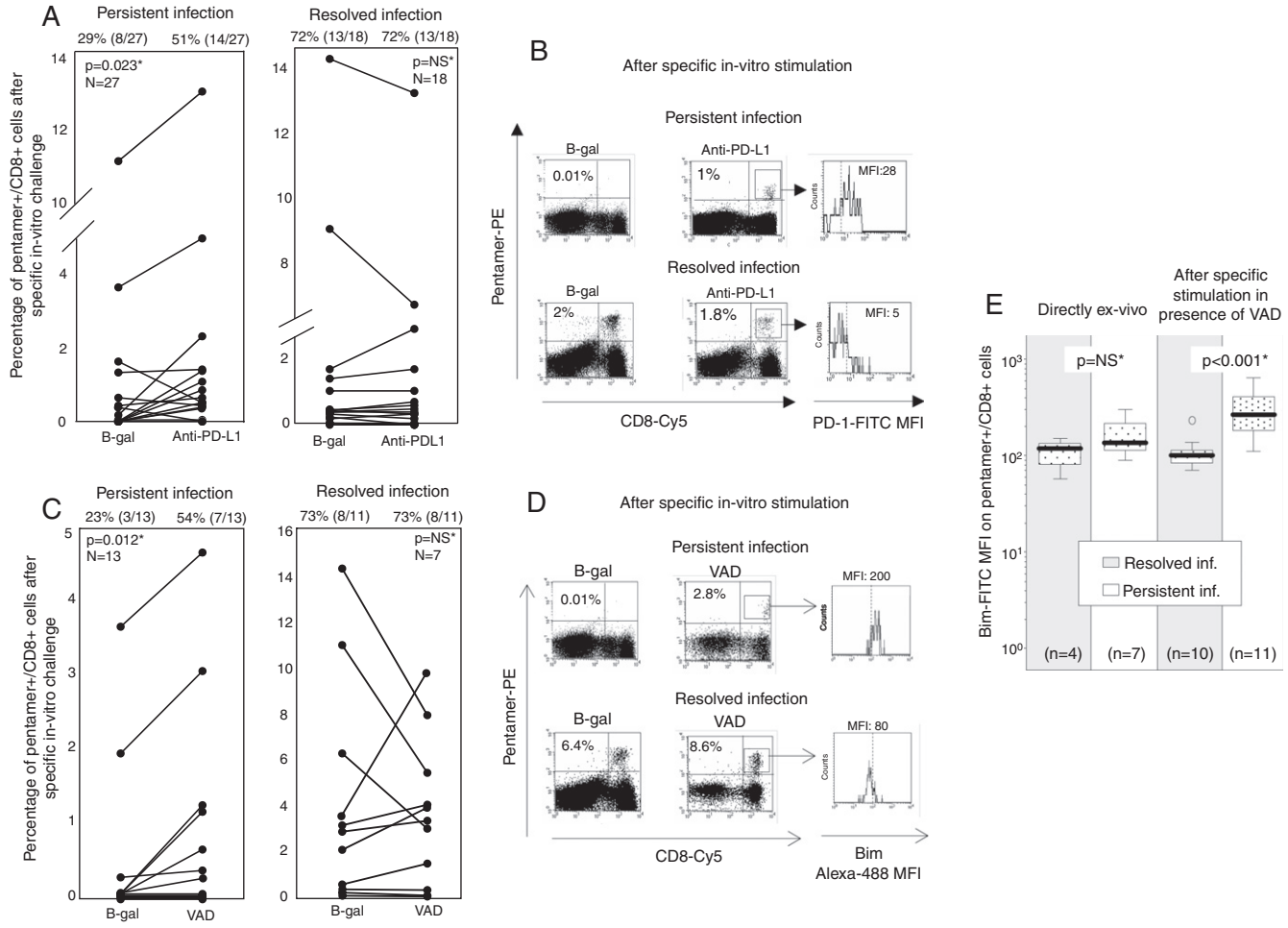

Fig. 6. Pentamer-binding cell proliferation ability restoration after blocking apoptosis and PD-1/PD-L1 pathway. (A) Plots showing the frequency of $CD8^+$/$Pentamer^+$ cells after specific *in vitro* challenge in presence of anti-PD-L1 mAb or β-galactosidase (β-gal) as control in PI and RI patients. ∗Wilcoxon test. NS: non significant. (B) Representative FACS® dot-plots of PBMC stained with PE-pentamer and CD8-Cy after *in vitro* stimulation in presence of β-gal or anti-PD-L1 mAb, and PD-1 FITC fluorescence intensity histograms of gated pentamer-binding cells after expansion from one RI and one PI case. Dash-line represents the upper limit of FITC staining for the isotype control. (C). Plots showing the frequency of $CD8^+$/$Pentamer^+$ cells after specific *in vitro* challenge in presence of z-VAD-fmk or β-gal as control in PI and RI patients. ∗Wilcoxon test. (D) Representative FACS® dot-plots of PBMC stained with PE-pentamer and CD8-Cy after *in vitro* stimulation in presence of β-gal or z-VAD-fmk, and Bim Alexa-488 fluorescence intensity histograms of gated pentamer-binding cells after expansion from one RI and one PI case. Dash-line represents the upper limit of Alexa-488 staining for the isotype control. (E) Box-plots showing the Bim Alexa-488 MFI in pentamer-binding cells directly *ex vivo* and after specific *in vitro* expansion in presence of z-VAD-fmk in RI and PI patients. ∗Mann–Whitney *U* test.

Table 4
Summary of the phenotypic and functional features of HCV-specific $CD8^+$ cells according to HCV control.

	PD-1 *ex vivo*	CD127 *ex vivo*	Epitope mutation	Expansion†	Expansion (αPD-L1)§	Expansion (zVAD-fmk)¤
Persistent infection						
PBMC*	(++)	(−)	Absent	Impaired	Increase	Increase
	(+)	(+)	Present	Non-impaired		
IHMC	(++)	(−)				
Resolved infection						
PBMC	(−)	(++)		Non-imparied	No change	No change

*In persistent infection two different peripheral HCV-specific CD8 + populations according to PD-1/CD127 phenotype were shown. (-) Low expression; (+) Intermediate expression; (++) High expression. Expansion: proliferation after specific *in vitro* challenge †without any treatment, §in presence of anti-PD-L1 mAb, and after treatment with ¤z-VAD-fmk. Ag: antigen. PBMC: peripheral blood mononuclear cells. IHMC: intrahepatic mononuclear cells.

geting the virus expressed a directly *ex vivo* $CD127^-$/$PD\text{-}1^+$ phenotype, as was expected. Therefore, therapeutic strategies focused on restoring HCV-specific CTL response should be directed against non-reactive $CD127^-$/$PD\text{-}1^+$ cells, which are the ones able to recognise the current infecting virus and potentially able to control the infection.

In the second part of our study, we tried to modify HCV-specific CTL reactivity through manipulation of PD-1 and CD127 pathways. To attempt to restore *in vitro* HCV-specific CTL reactivity in chronic patients, PD-1/PD-L1 pathway and IL-7 deprivation induced apoptosis were blocked. PD-1/PD-L1 interaction conducts to anergy by blocking the TCR signalling stream [6]. In fact, blocking this interaction with anti-PD-L1 mAb treatment resulted in a significant in-

crease of PI cases with HCV-specific CD8$^+$ cell expansion after antigen encounter as it has been previously observed by others [20,29]. Interestingly, not all dysfunctional T cells were rescued by blockade of the PD-1/PD-L1 pathway, suggesting that it is probably necessary to modulate other additional mechanisms in order to restore CTL reactivity, some of which have already been described [24,30,31]. Interestingly, a previous report shows that CD127 expression correlates inversely with the expression of several negative co-stimulatory molecules such as PD-1, suggesting that anergy and apoptosis are processes closely related [24] and, indicating that probably it is necessary to act in both pathways to obtain the HCV-specific CTL response restoration. Therefore, blocking intrinsic apoptosis pathway could be one of the necessary mechanisms to improve HCV-CTL reactivity as it has been shown in other persistent hepatotropic viral infection [23]. In chronic HBV infection, CD127$^-$ specific-CTL are prone to apoptosis due to the down-regulation of the antiapoptotic molecule Mcl-1 and the up-regulation of the apoptosis facilitator Bim, secondary to IL-7 deprivation [7,23]. In our study, by first time to our knowledge, HCV-specific CTL reactivity was significantly restored in chronic HCV infection through an anti-apototic *in vitro* treatment, using the irreversible pancaspase inhibitor zVAD-fmk. Interestingly, these cells displayed a high Bim expression, suggesting a similar apoptotic mechanism to the one described in chronic HBV infection [23]. Cytokine withdrawal on T cells results in activation of the mitochondrial apoptosis pathway, regulated by Bcl-2 protein family members. Bim pro-apoptotic activity is counteracted by the binding to Mcl-1[32], but in the case of Bim up-regulation this association would fail and apoptosis could be induced. As a result, our data could suggest that IL-7 deprived CD127$^-$ HCV-specific CTL up-regulate Bim expression during chronic infection, and this could explain the low T cell reactivity after antigen encounter due to apoptosis induction.

Consequently, strategies directed to block the pro-apoptotic effect of IL-7 deprivation should be designed to increase the effectiveness of CTL response restoration, in addition to blocking other negative co-stimulatory molecules. Short-term use of cyclosporine-A or FK506 could block the induction of the pro-apoptotic molecule Bim on CD127$^-$ cells [33]. This strategy could favour HCV-CTL restoration during anti-HCV treatment in combination with the standard of care.

5. Conclusions

In summary, we postulate that in persistent HCV infection, there are two different populations of peripheral HCV-specific CTL; PD-1$^+$/CD127$^-$ subset without expansion ability and prone to apoptosis and other one PD-1$^+$/CD127$^+$, characterised by maintaining proliferation capacity, although not targeting the current infecting virus. When HCV-specific CTL reach the liver, they acquire a PD-1^{++}/CD127$^-$ phenotype, which could predispose them to apoptosis and anergy. Therefore, strategies designed to block Bim-mediated apoptotic mechanisms on CD127$^-$ cells plus blocking of the PD-1/PD-L1 pathway could restore functionality of HCV-specific CD8$^+$ cells targeting the virus. To restore the functionality of these cells could be important to obtain a SVR, since reactive PD1$^-$/CD127$^+$ HCV-specific CTL are found in most of sustained responders.

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REVIEW

Role of T cell death in maintaining immune tolerance during persistent viral hepatitis

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Abstract

Virus-specific T cells play an important role in the resolution of hepatic infection. However, during chronic hepatitis infection these cells lack their effector functions and fail to control the virus. Hepatitis B virus and hepatitis C virus have developed several mechanisms to generate immune tolerance. One of these strategies is the depletion of virus-specific T cells by apoptosis. The immunotolerogenic liver has unique property to retain and activate naïve T cell to avoid the over reactivation of immune response against antigens which is exploited by hepatotropic viruses to persist. The deletion of the virus-specific T cells occurs by intrinsic (passive) apoptotic mechanism. The pro-apoptotic molecule Bcl-2 interacting mediator (Bim) has attracted increasing attention as a pivotal involvement in apoptosis, as a regulator of tissue homeostasis and an enhancer for the viral persistence. Here, we reviewed our current knowledge on the evidence showing critical role of Bim in viral-specific T cell death by apoptotic pathways and helps in the immune tolerance.

Key words: T cell death; Specific cytotoxic T lymphocytes; Hepatitis C virus immune tolerance; Apoptosis; Bcl-2 interacting mediator; Liver tolerance; Apoptotic pathways; Viral hepatitis

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INTRODUCTION

Hepatotropic, non-cytopathic viruses such as hepatitis B virus (HBV) and hepatitis C virus (HCV) behave as intracellular parasites. The activation of cellular immune response by priming of naïve specific CD4+ and CD8+ T cells in the lymph nodes is very important to control viral infection. However, the unique ability of the liver to retain and activate naïve CD8+ T cells leads to liver tolerance, by-passing normal activation in the lymph nodes. The continuous triggering of antigen presenting cells (APCs) in the sinusoids by the antigen-rich blood leads to peripheral tolerance to protect the liver tissue. This physiological feature can be used by hepatotropic viruses as a persistence mechanism. The depletion of liver activated CD8+ T cells is the critical part of the peripheral tolerance in HBV/HCV infection. The anticipated mecha-

nisms for immune tolerance in liver specific pathogens are linked to virus-specific T cells death. The vital role of pro-apoptotic molecule, Bcl-2 interacting mediator (Bim) in the death of the virus-specific T cells has been shown after intrahepatic T cell activation by hepatocytes[1], in chronic HBV and HCV infection[2,3]. Therefore, this review provides glimpse of the recent advances to understand the cellular and molecular mechanism involved on "T cell death" during viral hepatitis as a viral escape mechanism through the induction of a specific-immunotolerant status on the host.

VIRAL HEPATITIS

HBV and HCV viruses are two hepatotropic noncytopathic, human blood-born viruses. HBV is a small, enveloped DNA virus that undergoes a pro-viral state to persist in the host. HCV is an enveloped virus with a plus-strand RNA genome. It has been estimated that more than 350 million for HBV and 170 million people for HCV are infected. Approximately 80% of infections in HCV and > 90% of infected neonates, 5%-10% of infected adults in HBV succeed in establishing a chronic infection, with the potential for developing severe liver diseases such as cirrhosis and hepatocellular carcinoma[4,5].

Highly productive and replicative viruses such as HBV and HCV are associated with ineffective antiviral immunity during persistent viral infections. The complex ineffective immunity involves the functional deterioration of antiviral T cell responses and contraction of the size of this response. In persistent HBV/HCV infections, T cells are continuously challenged by high levels of viral antigens that eventually result in limiting the antiviral T cell response and ultimately leading to T cell exhaustion. This is a progressive process, starting with the deficiency in cytokine production, proliferation and survival[6], to end with physical deletion of specific antiviral T-cell populations[7].

Meticulously, cytotoxic T lymphocytes (CTLs) play a vital role in viral eradication[8] and in the pathogenesis of hepatitis[9-11]. A strong, multi-specific and long-lasting T-cell immune response emerges to be important for control of viral infection[12-14]. Appropriate, polyclonal, vigorous and multi-specific CTL responses can facilitate complete viral clearance, in which long-lasting protective T cell response is observed. However, specific CTL responses are usually not strong enough to eradicate the virus, hence contributing to persistent infection[15,16].

HBV and HCV are hepatotropic viruses that replicate in the liver. This organ features a unique immune tissue, where the deletion of antiviral T cell populations has been shown, being involved in local and systemic immune tolerance.

LIVER AS A FOUNDATION OF IMMUNE TOLERANCE

Liver situates at a hemodynamic convergence, receiving the splanchnic stream, which means an intense contact with exogenous antigens. This fact leads to the development of tolerance mechanisms to avoid inappropriate immune system activation, but it also allows antigen presentation by resident cells. Therefore, the liver is progressively more being recognized as an immune organ[17]. Liver sinusoids, hepatic arteries and portal venous carry blood containing digested nutrients and micro antigens from intestine, and as a primary metabolic organ, the liver produces multiple neo-antigens. All these molecules pass through sinusoids and finally are taken up and metabolized by different hepatic resident cells. The liver has acquired specialized mechanisms of immune tolerance to avoid the over reactivation of immune response against antigens that are metabolized in the liver. In fact, this process may be beneficial for inducing tolerance to liver grafts but also to the liver specific pathogens. Therefore, hepatotropic viruses exploit these immunotolerogenic liver features to persist. It is important to remind that the liver has the ability to retain and activate naïve CD8+ T cells ineffectively, in contrast to other lymphoid tissues. This fact may allow pathogens to escape from T cell mediated immunity and establish a persistent hepatic infection due to immune tolerance induction. This immunotolerant state can be reached by the development of T cell anergy but also by specific T cell deletion.

Uniqueness of the liver

The unique character of the hepatic tissue to tolerate liver allograft across major histocompatibility complex (MHC) mismatch in the pig without immunosupression was described by first time in 1969[17]. Later studies confirmed that this occurred because of the induction of immunological tolerance in the liver[18]. Initially, "graveyard theory" suggested that the exclusive ability of the liver to get rid of activated T cells, programmed to undergo apoptosis, was the root of the hepatic tolerance effect[19]. This theory proposed two functions of the liver as a T cell graveyard: (1) passive killer of the liver cells after their life cycle; and (2) efficient killer of the activated antigen specific T cells. According to this theory, T cell receptor (TCR) triggering by cognate antigen on TCR transgenic T cells leads to activation and accumulation of those cells in the liver and undergoes depletion of mature T cells[20].

The theory was again proved by Mehal *et al*[21] by indicating that the normal liver is a "sink" for activated T cells. The liver was perfused by T cells showing retention of activated, but neither resting nor apoptotic T cells[21]. Liver as a graveyard for activated T cells theory forced to believe that all the immune response in the liver would be silent; in spite of this, the presence of an effective virus specific T cells in patients controlling hepatic viral infections[22,23] could challenge this theory. Nonetheless, the removal of activated T cells by the liver cannot be excluded, as evidenced by the ability of liver allograft to rescue rejecting skin grafts[21], in which lately tolerising capacity of the liver for activated allo-specific T cells occurs. In some cases, the limited capacity of the liver to induce tolerance

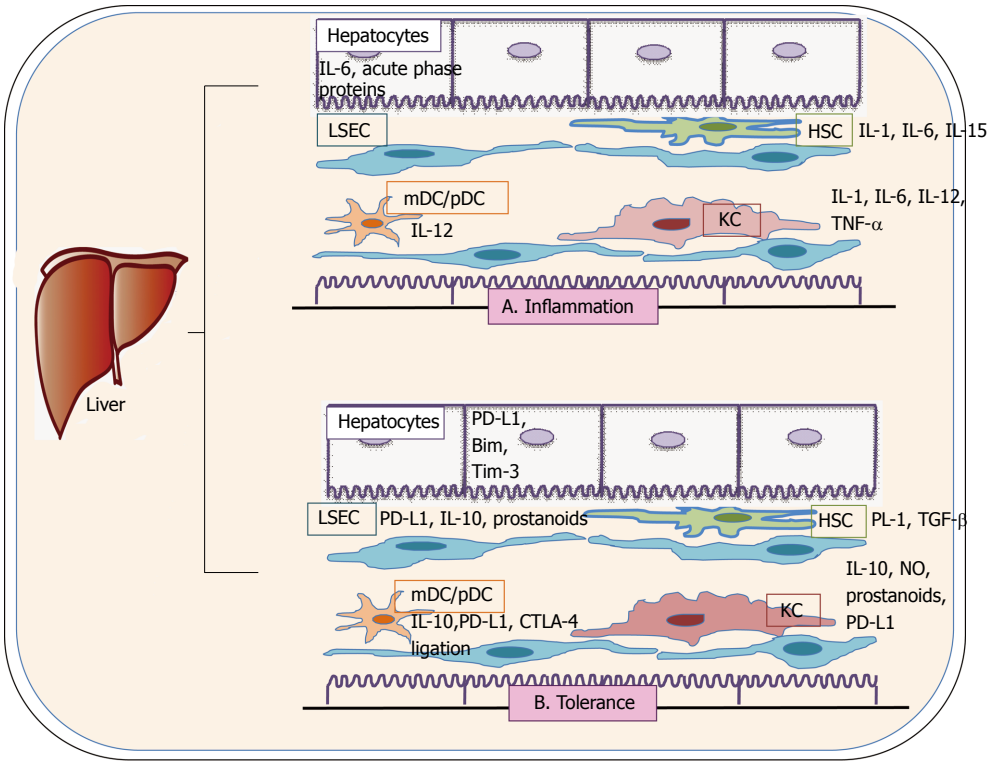

Figure 1 Collective illustration of the hepatic cells with inflammatory and tolerance activities by stimulation of different molecules or receptors. LSEC: Liver sinusoidal endothelial cells; KC: Kuffer cells; DC: Dendritic cells; HSC: Hepatic stellate cells; TNF: Tumor necrosis factor; IL: Interleukin; mDC: Myeloid dendratic cell; pDC: Plasmacytoid dendritic cell; PD-L1: Programmed death ligand-1; Bim: BCL-2 interacting mediator; Tim-3: T cell immunoglobulin mucin-3; CTLA-4: Cytotoxic T-lymphocyte antigen 4; TGF: Transforming growth factor; NO: Nitric oxide.

could be due to large number of activated T cells[24].

Naïve T cells activation in the liver

The site of T cell activation is a determinant of the outcome of an immune response in the liver[22]. Tolerance will occur when T cells are activated in the liver. On the other hand, an effective immune response will be generated, when T cells are activated in the lymph nodes. This model put forward the theory that tolerance during viral hepatitis could be the result of early deletion of antigen-specific T cells from the T cell repertoire in the liver[22]. Usually, naïve T cells are activated in secondary lymphoid organs with consequent up regulation of adhesion molecules and integrins expression, which can bind to endothelial layer of the target organ and ultimately direct T cells to the parenchyma[25]. Moreover, T cells are not able to interact with parenchymal cells easily and thus they are not usually activated in the solid organs. In spite of this, the situation in the liver is slightly different. Fenestrated endothelial layer in the liver makes available interactions between naïve T cells and liver cells[26]. It has been showed by MHC class I -restricted, hepatitis B surface Ag-specific CD8+ polyclonal CTL adoptively transferred into wide-spread antigen expressing transgenic mouse model, leading to retention of those cells within the liver[26]. Moreover, it has been shown that primary antigen-specific T cell can be activated in the liver independently of lymphoid tissues[27].

Liver APCs in tolerance

Retention, activation and tolerance of naïve T cells in the liver is the result of the action of resident liver cells, including liver sinusoidal endothelial cells (LSEC), Kuffer cells (KC), liver dendritic cells (DC), hepatic stellate cells (HSC) and hepatocytes. Their collective function in induction of inflammatory response and tolerance has been illustrated in the Figure 1.

Endocytosis specialist-LSEC can express MHC class I and Ⅱ, accessory CD80, CD86 and CD40 molecules. These features enable those cells to behave as potent APCs with the ability to activate both naïve CD4 and CD8 T cells as well as to cross-present exogenous antigen towards CD8 T cells[28]. However, LSEC primed naïve CD4+ T cells produce cytokines typical from Th0 rather than Th1 cells[29]. In addition, LSECs constitutively expressed ICAM-1, which helped in trapping of specific CD8+ T cells in the liver, resulting this process in activated T cell apoptosis[21]. Furthermore, the cross presentation of antigen by LSEC mainly leads to CD8+ T cells tolerance rather than immunity, demonstrating that LSEC-induced tolerance is an active and dynamic process[30].

Bone marrow derived and largest group of liver resident macrophages-KC mediate host resistance to infection. Interleukin (IL)-1, IL-6, IL-12 and tumor necrosis factor-α (TNF-α) pro-inflammatory cytokines released by KC[31] are involved in the inflammatory activities, whereas the nitric oxide, prostaglandin and IL-10 released by KC[29,32] down-regulate the production of pro-inflammatory cytokines and thereby may contribute to induction of hepatic tolerance. Furthermore, DC-induced antigen-specific T cell activation can be inhibited by KCs[29], which could also favor tolerance development. In addition, as in LSECs, KCs expressed ICAM-1 mediated trapping of specific CD8+ T cells in the liver resulting in activated T cell death[21].

Liver DCs are primarily located within periportal areas

and around central veins, which exert tolerogenic properties due to "immature" phenotype. The production of PD-1 and cytotoxic T lymphocyte antigen-4 (CTLA-4) by resting DCs, which are crucial negative co-stimulatory molecules, helps in inducing peripheral CD8+ T cell tolerance by inhibiting proliferation and cytokine production of liver infiltrating effector T cells[33]. In addition, liver generated DCs are more tolerogenic than DC in lymphatic tissue[34].

The role of HSCs in hepatic fibrosis includes stimulation of CD4, CD8+ T cells and NKT cells[35,36]. However, function of HSCs involves not only the inflammatory response[36], but also a tolerogenic role[37,38], which is the result of induction of T cell death[38] by intrinsic mechanism of immune inhibition. The HSCs regulate immune modulation by inducible B7-H1 expression, an inhibitor molecule of B7 family, resulting in T cell apoptosis induction.

Hepatocytes are also capable of activating naïve CD8+ T cells[39,40] and their interactions with CD8+ T cells may occur through LSEC fenestrations[38]. However, hepatocytes fail to promote activated CD8+ T cells survival, leading to an impaired T cell activation[39]. In addition, hepatocyte-activated T cells *in vitro* acquired activity and secrete cytokines but both levels are not constant and T cells consequently appeared to die by passive mechanisms[41]. Furthermore, infiltrating CD4+ T cells differentiate into a less inflammatory phenotype due to the interaction with MHC Ⅱ-expressing hepatocytes, which also helps to abrogate antiviral CD8+ T-cell response and viral clearance[42], which conclude in the tolerance during infection. It has been already proved that T cells activated by hepatocytes undergo premature death[43], whereas naïve CD8+ T cells priming by DC in the lymph nodes acquired effector functions in the liver.

The site of primary T cell activation could also induce emperipolesis of CD8+ T cells in the liver[43], which leads to non-apoptotic, destruction of these CD8+ T cells after degradation by lysosomal proteolytic enzymes. This distinct form of emperipolesis has been termed as "suicidal emperipolesis" (SE)[44]. Benseler *et al*[44] suggested that SE is a significant mechanism by which death of activated naïve CD8+ T cells occur in the liver within the first few hours before T cells are able to divide and expand. It is also involved in maintenance of tolerance, which is reinforced by break of tolerance in immune-mediated liver damage by treatment of wortmannin[44], inhibitor of phosphoinositide 3-kinases that blocks emperipolesis. Therefore, SE is an extremely efficient mechanism, able to rapidly delete T cells.

T cell stimulation in the liver encourages tolerance by using mechanisms such as, immune divergence[45], generation of regulatory T cells[46], T cell anergy[47] and T cell death[1]. Undeniably, hepatic tolerance can explain the elevated frequency of viral persistence during hepatotropic virus infections[1]. Although there are evidences showing that most infectious microorganisms are promptly removed from the liver, a favorable situation for evading immune responses occurs in some viruses, leading to the triumph of certain pathogens such as HBV and HCV. Till date, there are two main mechanisms by which HBV and HCV could successfully escape from CTL action: escape mutant generation, and immunosuppressive effects exertion (effector T cell exhaustion and T cell death by apoptosis)[2,48-50]. Among these mechanisms involved in viral hepatitis persistence, new advances on the role of T cell death induction have been obtained recently and our review in the apoptosis role, paying special attention to the last new insights in this issue will be discussed in the following pages.

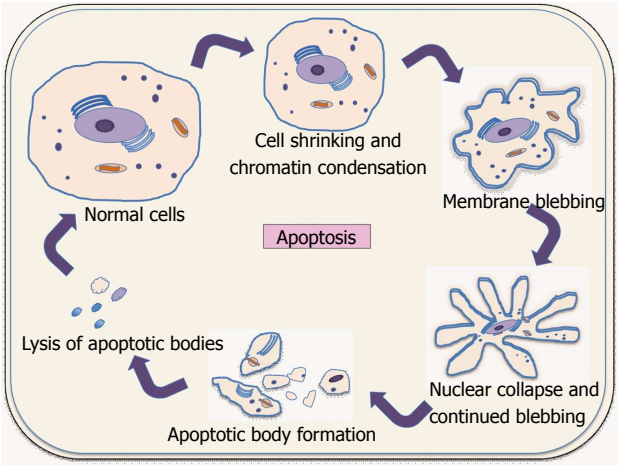

Figure 2 Apoptosis-programmed cell death.

APOPTOSIS

A normal cellular process involving physiologically relevant cell death and deletion of unwanted cells is called apoptosis. Apoptosis is essential for cell selection, tissue homeostasis, morphogenesis, and host defense in multicellular organisms. A cell that undergoes apoptosis dies neatly, without damaging its neighbors. The apoptotic signals give rise to activate various proteins and follow a specific classical caspase chain reaction set activation[51]. Quickly and neatly dismantlement process includes membrane blebbing with shrinking of the cytoplasm and condensation of the nucleus. Phagocytic cells begin to pick up the apoptotic bodies, preventing the release of cellular content and ultimately avoiding inflammation[52] (Figure 2). Apoptosis occurs by two mechanisms: active and passive mechanism. No presence of antigen gives a signal for termination of immune response by passive apoptotic mechanism (intrinsic pathway). On the other hand, the ligation of Fas (CD95) and TNF receptors-"death receptors" triggered apoptosis lead to active mechanism of apoptosis (extrinsic pathway). Briefly, apoptosis mechanisms involve a family of cysteine proteases, called caspases. These molecules are synthesized in the cell as inactive precursors, or pro-caspases for self-protection against accidental death, which are usually activated after receiving proper trigger by cleavage (Figure 3). Structurally, pro-caspases contain three domains: N terminal prodomain, a large subunit

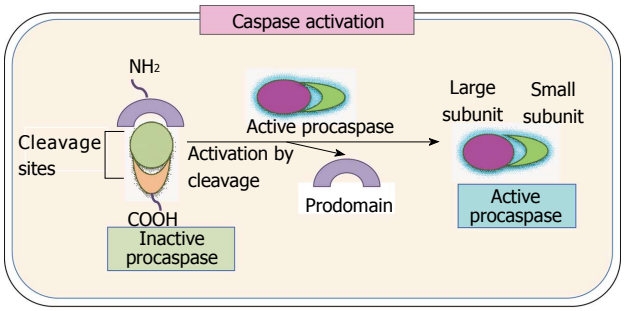

Figure 3 Caspase activation: Inactive proenzyme (procaspase) activated by proteolytic cleavage by another member of caspase family and cleaved two fragments associate to form the active site of the caspase.

and a small subunit. After activation, the active caspase enzyme is formed by heterodimerization of small and large subunits[43]. Moreover, active caspase molecules are ready to cleave target proteins such as structural or signaling proteins and other effector caspases, preventing other proteins cleavage randomly[52].

Extrinsic pathway

The extrinsic pathway initiates from outside the cell through triggering the activation of transmembrane "death receptors" that are members of the TNF receptor gene superfamily. Members of this receptor family bind to extrinsic ligands known as pro-apoptotic ligands[53] and transduce intracellular signals that ultimately result in the destruction of the cell[54,55]. To date the most well characterized ligands of these receptors are FasL, TNF-α, Apo3L and Apo2L and corresponding receptors are FasR, TNFR1, DR3 and DR4/DR5, respectively[55-57]. The signal transduction of active cell death process involves several caspases. Activated caspases have an effect on several cellular functions as part of the process that results in the death of the cells[53].

The signal transduction of mitochondrial-independent active cell death process involves binding of a pro-apoptotic ligand (such as FasL) with its receptors (Fas) on the surface of a target cell. The cytosolic tail of receptors contains a death domain, which when activated, binds to an adaptor protein, which in turn recruits the specific procaspase-8 and -10 and activates them by proteolytic cleavage[58] that finally initiates the proteolytic caspase cascade leading to apoptosis. Activated caspase 8 triggers the caspase cascade *via* two different pathways, leading to cell death. In type 1 apoptosis, such as in lymphocytes, caspase 8 activates caspase 3 whereas in type 2 apoptosis, like in hepatocytes and pancreatic cells, caspase 8 activate the pro-apoptotic molecule Bid and go ahead for apoptosis *via* the disruption of mitochondrial membrane and cytochrome C release[59] (Figure 4). The T cell death by type 1 and type 2 Fas induced apoptosis fate is decided by the ratio between proteolytically activated effector caspases, X-chromosome linked inhibitor of apoptosis protein and proto-typical effector caspase substrate inhibitor of caspase-activated DNase. Interestingly, HCV specific intrahepatic lymphocytes contribute to bystander killing *via* Fas-FasL interaction[60], which support the fact that the liver facilitates liver-trapped activated T cell apoptosis[61].

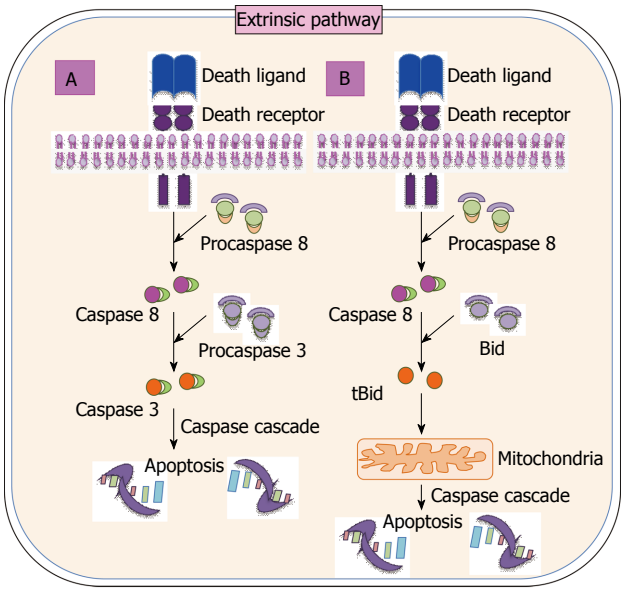

Figure 4 Extrinsic pathway. A: Mitochondria-independent extrinsic pathway: Fas-FasL ligation strikes to recruit pro-caspase 8 activation and induction of caspase cascade by caspase 3 leading to apoptosis; B: Mitochondria-dependent extrinsic pathway: Fas-FasL ligation trigger to activate the pro-caspase 8, which cleave Bid (pro-apoptotic Bcl-2 family molecule) to form truncated Bid (tBid). Then, mitochondrial dependent cell death begins with tBid.

Intrinsic pathway

The intrinsic or mitochondrial pathway is initiated within the cell, involving non-receptor-mediated intracellular signals and inducing activities in the mitochondria that initiate apoptosis. DNA damage, loss of cell-survival factors or other types of severe cell stress causes the induction signal for the intrinsic pathway. This passive death process pivots on the balance of activity between pro- and anti-apoptotic signals of the B cell lymphoma 2 (Bcl-2) family proteins[62]. This balance is maintained by regulation of the permeability of the mitochondrial membrane and by the pro- or anti-apoptotic signal that will be released inside the cell[63]. Following mitochondrial permeabilization, the intrinsic pathway divides into two pathways: Apoptosis protease-activating factor-1 (Apaf-1) dependent and Apaf-1 independent pathway. In Apaf-1 dependent pathway, release of cytochrome c from mitochondria, by triggering the pro-apoptotic Bcl-2 family member[64], and ATP activate monomer inactive Apaf-1 proteins by a conformational change, leading to form a heptamer of Apaf-1 molecules called apoptosome[65]. Apoptosome allows activation of pro-caspase 9, which consequently triggers the caspase cascade[66]. On the other hand, in Apaf-1 independent pathway, permeabilization of mitochondrial membrane release DIABLO like proteins, which activates effector caspases by provoking inhibitors of apoptosis proteins[67] and triggers caspase cascade[68] (Figure 5).

The balance of pro- and anti-apoptotic proteins main-

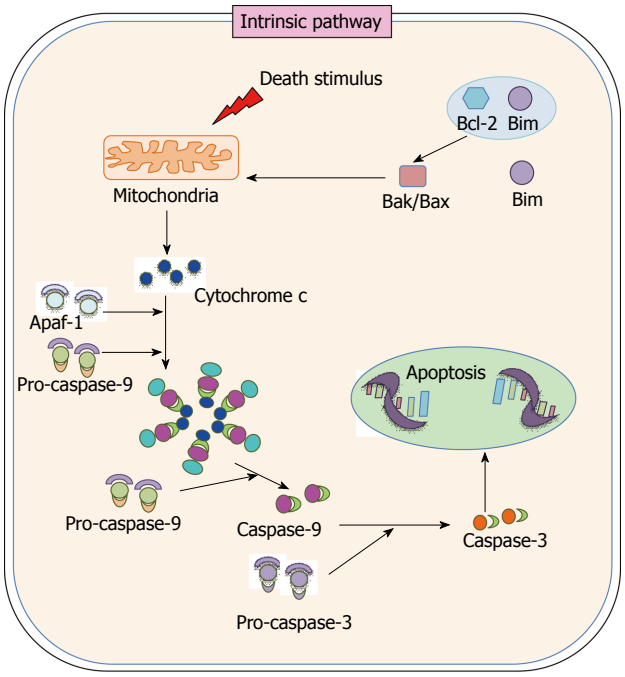

Figure 5 Intrinsic pathway. Death stimulation up regulates Bcl-2 interacting mediator leading to the separation from Bcl-2, favoring the activation of Bax, Bak, which form pores in the mitochondrial membrane leading to release of cytochrome c. Cytochrome c with Apaf-1 and procaspase 9 participate in the formation of apoptosome, which activate caspase 9. Caspase-9 activates caspase 3 after cleavage of pro-caspase-3. That caspase-3 triggers to induction of caspase cascade and cell death. Apaf-1: Apoptosis protease-activating factor-1. Bim: Bcl-2 interacting mediator.

tains the apoptotic activity[69]. The Bcl-2 family members regulate mostly neglect or intrinsic pathway. This family is subdivided into three groups of proteins on the basis of their functions and the number of Bcl-2 homology (BH) motifs included in their primary structure; first group: "anti-apoptotic multidomain" members, such as Bcl-xL, have four BH domains (BH1 to BH4) which inhibits apoptotic process. Other two groups of "pro-apoptotic multidomain" members, which are Bax-like proteins and "BH3-only" proteins[70]. Bax-like proteins possess threee BH domain (BH1 to BH3), including Bax, Bak, and Bok, which are referred as death effector members. BH3-only members contain BH3 domain, including Bim, Bad, Bik, Puma, Noxa and Bid and are known as messengers of death. In addition, C-terminal transmembrane (TM) fragment is thought to confer anchorage to mitochondrial membranes, which is also possessed by most multi-BH members and several BH3-only proteins.

Three models (Figure 6) have been postulated by which the BH3 family promotes passive cell death in which Bax and Bak bind directly or indirectly with cell death sensitizer (*e.g.*, Bad, Bik) and activators of cell death (*e.g.*, Bim, tBid). The direct activation model proposes that sensitizer BH3-only proteins displace the activator BH3-only proteins from the anti-apoptotic proteins to promote apoptosis. Anti-apoptotic proteins inhibit the activator BH3-only proteins but not Bax and Bak to suppress apoptosis. In the displacement model, Bax and Bak are sequestered by anti-apoptotic proteins for cell survival and constitutively active in cells. BH3-only proteins play the sensitizer role and inhibit their respective anti-apoptotic proteins to promote apoptosis. The third model, called embedded together model, highlights the interactions occurring in and on membranes, which were not explained by direct activation and displacement model. In embedded together model, Bcl-2 family proteins insert into and change their conformations according to their functions in membrane[71]. The predominantly studied messenger death molecule, Bcl-2 interacting protein (Bim) will be focused further.

BIM

Bim/Bod is a pro-apoptotic protein belonging to the BH3-only group of Bcl-2 family members and is being called the "ghost" molecule or "suicide" molecule, which enables cells to expire gracefully. Two independent studies discovered Bim as a Bcl-2 binding protein and Mcl1-binding protein in 1998[72,73]. Bim induces apoptosis by binding to and antagonizing anti-apoptotic members of the Bcl-2 family. The Bim interactions have been observed with Bcl-2 family members, such as Bcl-2, Bcl-xL, Mcl-1, Bcl-w, *etc*[72,73].

Bim is a well-known pivotal initiator of apoptosis in thymocyte-negative selection[74]. Bim has 19 Bim isoforms including three major isoforms, which have distinct sizes and pro-apoptotic activities in the mammals, caused by alternative splicing: BimEL (extra long), BimL (long) and BimS (small)[73]. The shortest form, BimS, is the most potent and is generally only transiently expressed during apoptosis[73]. The other two isoforms are sequestered to the dynein motor complex, and apoptotic activity of these longer isoforms is regulated by phosphorylation[75,76], which is triggered by environmental stress, resulting in its dissociation from the dynein complex and increasing apoptotic activity.

Expression of Bim is up regulated in human T cells in response to TCR-triggering by protein kinase C and calcineurin pathways[77]. Nevertheless, there are other mechanisms involved in Bim up-regulation during chronic infection, such as the effect of certain cytokines. In fact, in a persistent viral infection animal model, Bim-mediated apoptosis correlates with low IL-7 receptor expression on specific T cells[78].

The regulation of Bim expression at transcriptional level in growth factor deprivation and in endoplasmic reticulum stress has observed by the class O fork-head box transcription factor (FOX03A) and transcriptional factor CEPB-α respectively[79,80]. Post-transcriptional phosphorylation of Bim can also regulate its function. Phosphorylated Bim is targeted for proteasomal degradation and avoid its interaction with Bax, thus maintaining cell existence[81,82]. The signaling adaptor TNFR-associated factor 1 (TRAF1) negatively correlates with Bim and it contributes to CD8 T cell-mediated control of chronic viral infections. In addition, linking between survival

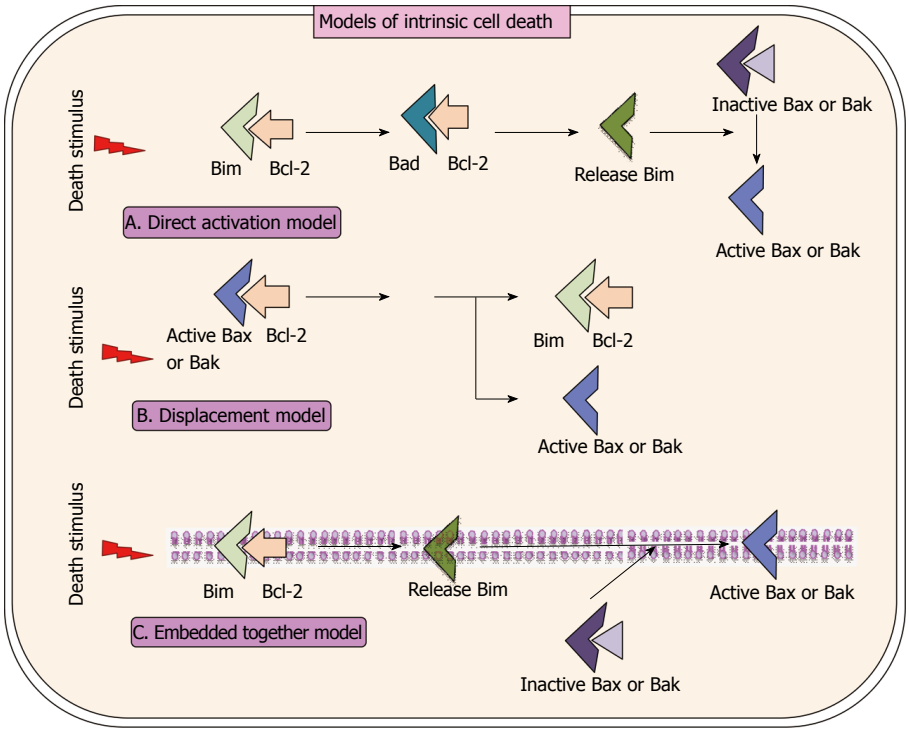

Figure 6 Models for intrinsic cell death. A: Direct activation model postulates Bcl-2 interacting mediator (Bim) is required for activating Bax and Bak. Anti-apoptotic proteins inhibit BH3-only proteins to suppress apoptosis, but not Bax or Bak. Replacement of Bim to sensitizer BH3-proteins from the anti-apoptotic proteins occurs to promote apoptosis; B: The displacement model proposes that anti-apoptotic proteins for cell survival must sequester constitutively active Bax and Bak in cells. Bim inhibits their respective anti-apoptotic proteins by playing sensitizer role to promote apoptosis; C: Embedded together model highlights the active role of the membrane, which is not defined in direct activation model and displacement model. Bcl-2 family proteins insert into and change their conformations that dictate their functions at the membrane. Sensitizer BH3-only proteins relocate the activator BH3-only proteins and Bax/Bak from the anti-apoptotic proteins to endorse apoptosis. Activator BH3-only proteins recruit Bax to the membrane to induce mitochondrial outer membrane permeabilization and apoptosis. These reversible interactions are directed by equilibrium constants that are depended on the concentrations and interactions of the proteins with each other and with membranes.

effects of TRAF1 and TRAF1-dependent Bim down-modulation has been shown in CD8 T cells[83-85]. TRAF1 is particularly vanished from virus-specific CD8 T cells during the chronic human immunodeficiency virus and lymphocytic chorio-meningitis virus (LCMV) infection[86].

Bim plays a vital role in the immune system, in bone biology and in tumor-genesis by inducing apoptosis[87]. Bim in T cells, B cells, neurons and many other cell types can trigger apoptosis[87]. Gene targeting in mice for the important region for apoptosis, BH3 region, uncovered the important physiological role in Bim[88]. In fact, in the absence of Bim leukocytes in blood as well as in LNs, thymus, spleen were high in number[88]. The role of Bim in apoptosis has been revealed in Bim-/- thymocytes, which were more resistant to apoptosis after different apoptotic treatment such as ionomycin, taxol, γ irradiation[88].

DEATH OF ACTIVATED T CELLS BY BIM

The liver is having a property that might explain its role in inducing tolerance due to its recognition as an alternative primary activation of CD8 T cells site. The phenotype of activated CD8 cells in the liver was the same as in lymph nodes. However, liver-activated CD8 T cells displayed poor effector functions and a unique $CD25^{low}$ $CD54^{low}$ phenotype, which was associated with increased expression of the Bim and caspase-3, demonstrating that these cells are programmed to apoptosis following intrahepatic activation. Strikingly, Bim deficient T cells survived following intrahepatic activation[1]. Therefore, the phenotype and fate of naïve CD8 T cells activated by hepatocytes *in vivo* could explain the death penalty role of Bim in chronic hepatotropic viral infection[1]. The distinct phenotype can be due to the lack of co-stimulatory molecule expression on hepatocytes[43]; however the treatment with IL-2 or anti-CD28 antibodies could rescue hepatocyte-activated cells from death[41].

Lymphocyte fate deciding pathways synergize to kill activated T cells in chronic herpes simplex viral immune responses, whereas death of activated T cells in acute immune responses relies only on the mitochondrial pathway involved only Bim with no contribution by Fas, which showed critical overlapping roles for Fas and Bim in T cell death during immune response shutdown, leading to immune tolerance[23].

BIM IN HEPATITIS

Bim has been shown to be important for CD8 T cell viability during chronic LCMV infection in mice[89]. In this study, in Bim mutated mice, Bim mutation almost completely blocked the deletion of cognate antigen specific CD8 T cells in liver during chronic viral infection. Bim has a critical role in maintaining naive and memory T cells in LCMV infection[90]. In another study, it has been shown that a defect in apoptosis dramatically not only enhances the antigen-specific memory T cells but also increased the number of virus-specific CD4+ T cells in the lymph nodes following acute LCMV infection, compared to the parental genotypes or wild type mice[91]. Therefore, the loss of both Bim and Fas caused the increase in memory T cells in acute LCMV infection[91]. The Bim role has been demonstrated in the development of LCMV-induced, T cell-mediated hepatitis by controlling the apoptosis of both T cells and hepatocytes[92].

Bim attrition of virus specific CTLs during HBV

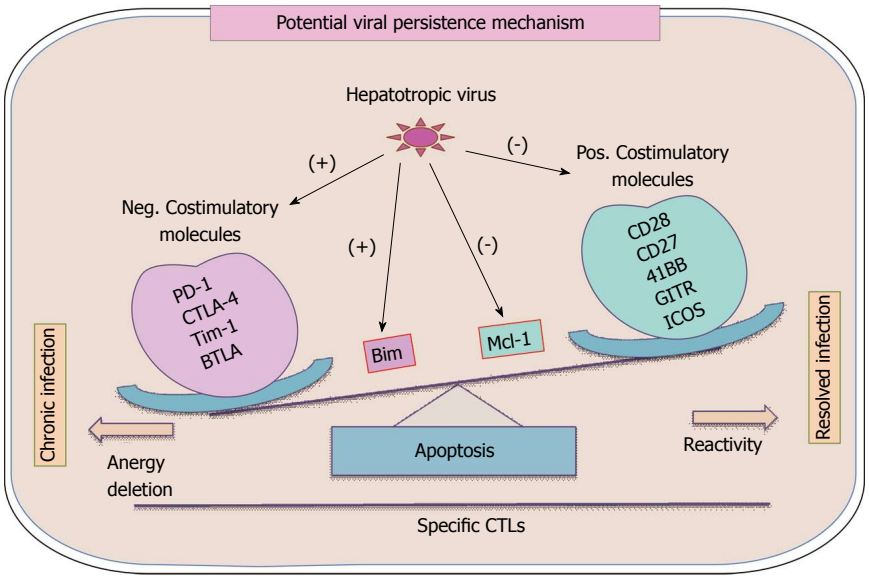

Figure 7 Balance between co-stimulatory/apoptotic molecules and viral-specific cytotoxic T lymphocytes reactivity according to infection outcome. Neg.: Negative; Pos.: Positive; CTLs: Cytotoxic T lymphocytes; (+): Possible molecules induced by viral infection; (-): Possible molecules down-regulated by viral infection; BIM: Bcl-2 interacting mediator; Mcl-1: Myeloid cell leukemia sequence-1.

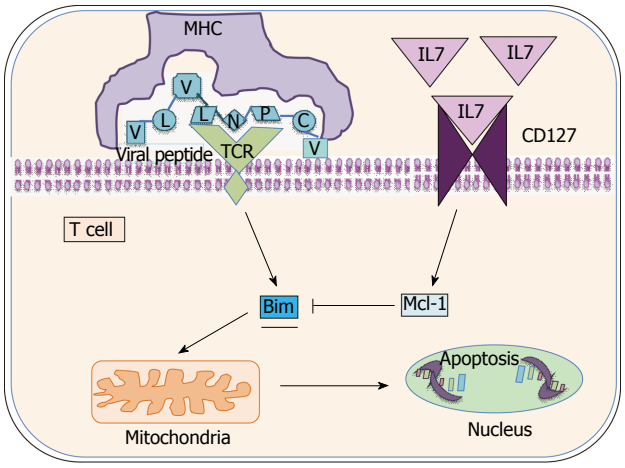

Figure 8 Cell survival marker CD127 modulates Bim and myeloid cell leukemia sequence-1 expression on hepatitis C virus-specific cytotoxic T lymphocytes after cognate antigen stimulation. Misbalance of Mcl-1/Bcl-2 interacting mediator (Bim) triggers to apoptosis of hepatitis C virus specific cytotoxic T lymphocytes. TCR: T cell receptor; Mcl-1: Myeloid cell leukemia sequence-1.

infection has also been confirmed[3,93]. The gene expression profile in HBV infection showed different patterns of gene expression on HBV-specific CD8+ T cells according to viral control. Bim was one of the up-regulated genes in HBV-specific CD8+ T cells from patients with chronic HBV infection. Blocking Bim-mediated apoptosis improved recovery of HBV-specific CD8+ T cell function[3]. Furthermore, the elevated apoptosis has been observed not only with Bim tolerogenic phenotype, but also with co-inhibitory signals through CTLA-4[93] or T cell-intrinsic transforming growth factor-β[94].

As discussed earlier, robust CD8 responses are essential to control HCV infection. However, in HCV chronic infection, HCV specific CD8 are depleted by Bim mediated attrition, and remaining cells are functionally exhausted. The cell survival factor CD127 counteracts the induction of apoptosis after antigen encounter through myeloid cell leukemia sequence-1 (Mcl-1) expression and Bim down-regulation[95] after the cognate antigen recognition by TCR. Similarly, our group has shown in previous work, HCV-specific CTLs displayed a high Bim expression in persistent infection respect to resolved infection patients[2], suggesting a similar apoptotic mechanism to the one described in chronic HBV infection.

The procedure of T cell death during chronic viral infection is determined by a carefully balanced and complex group of pro- and anti-apoptotic proteins of the Bcl-2 family, such as Bim and Mcl-1[96] (Figure 7). Interestingly, persistent hepatotropic viral infection is characterized by continuous TCR triggering and CD127 down-regulation on viral-specific CTLs[97], which could favor Bim up-regulation. In addition, it is well known that Bim is clearly involved in intrahepatic specific-CTL apoptosis in animal models[1]. Furthermore, Bim pro-apoptotic effect is blocked by the action of Bcl-2 family anti-apoptotic proteins such as Mcl-1 and Bcl-2[78,98], clearly pointing out that T cell death also depends on the anti-apoptotic protein expression. Bearing in mind all these facts, recently our group has suggested a model to explain specific CTL deletion during persistent hepatotropic viral infection (Figure 8). This model shows that CD127 phenotype modulates Bim and Mcl-1 expression on virus-specific CTLs, leading to Mcl-1/Bim imbalance during persistent infection, which impairs T cell reactivity and suggesting that restoration of T cell function could occur by correcting the levels of Mcl-1 and Bim expression.

In our work, Bim up-regulation has been observed on $CD127^{low}$-expressing HCV-specific CTLs but not on $CD127^{high}$ cells after antigen encounter, suggesting that TCR triggering can only lead to Bim up-regulation in absence of IL-7 stimulation on HCV-specific CTLs. Nevertheless, Bim level is not enough to lead to T cell apoptosis. Our data also showed the Mcl-1/Bim ratio could decide the fate of the activated T cells by sequestration of experienced $CD127^{low}$/$Mcl\text{-}1^{low}$-expressing T cells to the liver and subsequent Bim up-regulation after antigen encounter due to the absence of IL-7 stimulus[99]. Finally, Bim

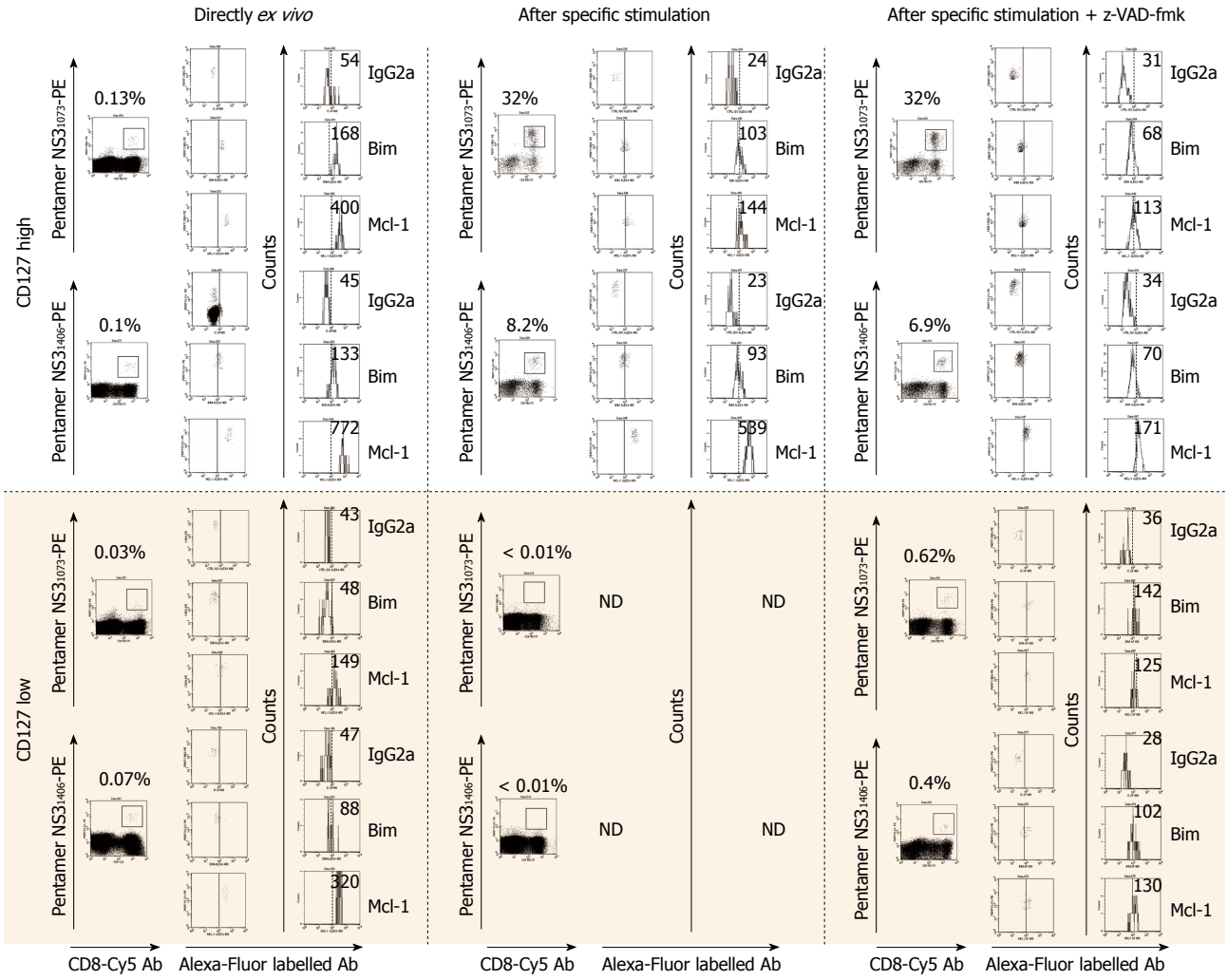

Figure 9 Figure illustrated the FACSan® dot plots and histograms from peripheral blood lymphocytes from two hepatitis C virus patients with different CD127 expression on hepatitis C virus-specific cytotoxic T lymphocytes (CD8+/Pentamer+ cells). The different plots show the Bcl-2 interacting mediator (Bim) and myeloid cell leukemia sequence-1 (Mcl-1) expression directly *ex vivo* and after specific stimulation on peripheral CD8+/pentameter+ cells according to CD127 level. The figure on the top of the dot-plots represents the frequency of penatmer+ cells out of total CD8+ cells. The figure in the upper right corner in the histogram plot represents the MFI for Bim and Mcl-1 staining. The continuous and dashed line in the dot-plots and histograms represents the cut-off point to consider a staining positive according to the negative control. ND: Not done due to lack of pentamer+/CD8+ proliferation after specific stimulation.

would be released freely to activate Bax, due to the low level of the anti-apototic protein Mcl-1 during chronic HCV infection. Consequently, CD127 level play a central role in hepatotropic virus-specific CD8+ T cell apoptosis by regulation of Mcl-1 expression *in vivo* and by Bim modulation after antigen encounter, which is checked by T cell reactivity restoration and Mcl-1/Bim phenotype on CD127^{low} specific CTLs after apoptosis blockade (Figure 9), that suggested a link between apoptosis after TCR triggering and low CD127 expression on experienced specific CTLs during persistent infection that could be related to Mcl-1/Bim imbalance.

Therefore, CD127 phenotype modulates Bim and Mcl-1 expression on specific CTLs and this affect to T cell reactivity through apoptosis regulation. Specifically, during chronic infection, Mcl-1/Bim imbalance could be involved on CD127^{low} specific CTL hyporeactivity, but it could be overcome by blocking apoptosis.

For control of hepatotropic viral infection is essential to develop a robust viral-specific cellular response. However, during chronic infection this response is altered, showing a pro-apoptotic phenotype due to the deprivation of IL-7 secondary to the low expression of CD127. Recently, it has been investigated that TRAF1 is a signal adapter for positive co-stimulatory receptors whose level depends on the action of IL-7 and inhibits the expression of the pro-apoptotic molecule Bim[86]. Therefore, in situations of deprivation of IL-7, action of TRAF1 could be impaired, favoring an imbalance between anti- and pro-apoptotic molecules. On the other hand, in an experimental model, IL-7 deprivation during stressing conditions leads to Mcl-1 down-regulation on T cells, conducting to T cell death that could be avoided by IL-7 treatment[100]. Consequently, strategies directed to block the pro-apoptotic effect of IL-7 deprivation should be designed to increase the effectiveness of CTL response restoration, by enhancing the TRAF1 and Mcl-1 expression level that could restore Bim/Mcl-1 balance. On of those strategies

could be short-term use of cyclosporine-A or FK506 could block the induction of the pro-apoptotic molecule Bim on CD127$^-$ cells[77]. This strategy could favor specific-CTL restoration during anti-viral treatments in combination with the standard of care. Another possible strategy to restore hepatotropic virus-specific CTL reactivity during chronic infection could be the administration of IL-7, in order to increase the stimulation of the reduced number of IL-7R molecules on specific CTLs, to modulate the balance between Bim and Mcl-1. In fact, in an animal model of cytotoxic T cell exhaustion, IL-7 treatment resulted in amplified cytokine production, increased T cell effector function, and viral clearance[101].

CONCLUSION

The deletion of hepatitis virus-specific CD8+ T cells is likely to represent the deregulation of the Bim pro-apoptotic pathway. The balance between pro- and anti-apoptotic molecules is critical for cell survival. The unavailability of appropriate survival marker modulates Bim and Mcl-1 expression on virus hepatitis-specific CTLs and this affect to T cell reactivity through apoptosis regulation. The level of those molecules is regulated by CD127 (IL-7R) expression, which is down-modulated during persistent infection. Consequently, Mcl-1/Bim imbalance could be the reason for the deletion of virus hepatitis-specific T cells, but it could be overcome by interruption of apoptosis. The interruption of this tolerizing mechanism may provide a new strategy to restore the balance between apoptotic molecules in order to achieve viral specific T cell immunity, as a future treatment strategy of chronic viral hepatitis.

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HBV & HCV Immunopathogenesis

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1. Introduction

Hepatitis B and C (HBV&HCV) viruses are two hepatotropic non-cytopathic viruses able to evade immune system efficiently as mechanism to persist in infected hosts. To fight against a viral infection the host displays two kinds of immune responses: the innate and adptive responses. The innate response is the first immunological barrier and it is essential in cytopathic viruses. This response limits viral spreading but also acts as adaptive response activator through antigen presentation to viral specific cells. Adaptive response is the second line in the immunological defense. It plays a major role in non-cytopathic viral infections because this type of viruses behaves as an intracellular parasite and they remain occult to the innate system.

1.1 General features of Innate Immune response

The liver is a unique anatomical and immunological site in which antigens-rich blood from the gastrointestinal tract is passed through a network of sinusoids and scanned by antigen-presenting cells and lymphocytes. It is selectively enriched in macrophages (Kupffer cells), natural killer cells (NK) and natural killer T cells (NKT) which are key components of the innate immune system (Racanelli & Rehermann, 2006).

Innate immunity generally plays a role immediately after infection to limit the spread of the pathogen and to activate the adaptive immune response (Guidotti & Chisari, 2006). Complex interplay between innate and adaptive immunity is the key for the resolution of acute infections. Innate response is induced after host recognition of common molecular patterns expressed by viruses, immediately after primoinfection, and providing a mandatory environment for triggering efficient adaptive immune responses. During hide and seek game of virus and host, one or more viral products get exposed and recognized by early immune response. This starts anti-viral control through direct cytopathic mechanisms (Koyama *et al.*, 1998), antiviral effect by producing IFN type I (IFN-alpha/beta) by infected cells (Samuel, 2001), and activation of the cellular component of the innate immune system as natural killer (NK) cells and natural killer T (NKT) cells (Biron *et al.*, 1999).

Production of type I IFNs can be triggered directly by virus replication through cellular mechanisms that detect the presence of viral RNA or DNA (Alexopoulou *et al.*, 2001), while NK cells are activated by the recognition of stress-induced molecules and/or the modulation of the quantity of major histocompatibility complex (MHC) class I molecules on the surface of infected cells (Moretta *et al.*, 2005).

NK and NKT cells can be rapidly recruited to the site of virus infection and have the potential to recognize infected cells before MHC class I expression is significantly induced on the cell surface. Activated NK and NKT cells may participate in disease pathogenesis directly, by killing infected cells, and indirectly, by producing soluble factors that have antiviral activity, recruiting inflammatory cells into the infected tissue and shaping the adaptive immune response (Biron *et al.*, 1999).

1.2 General features of adaptive immune response

Non-cytopathic viruses behave as intracellular parasites which are hidden to the immune system. They are not usually highly infectious but produce long-lasting diseases that allow them to spread the infection along the time. The host-virus relationship is a dynamic process in which the virus tries to decrease its visibility, whereas the host attempts to prevent and eradicate infection with minimal collateral damage to itself (Nowak & Bangham, 1996).

To control non-cytopathic viral infections, it is necessary the activation of the adaptive immune system, and especially the cellular immune response. Naïve specific CD4+ and CD8+ T cells are primed by dendritic cells in the lymph nodes. Once these cells become activated, they change the phenotype into effector cells and migrate to the infected tissue, attracted by the chemokines produced by the parenchymal cells. Primed specific CD4+ cells are essential to allow the adequate activation of specific cytotoxic T cells by secretion of Th1 cytokines (Larrubia *et al.*, 2009a). This is very important because specific cytotoxic T lymphocytes play a major role in spontaneous infection resolution. These cells are able to recognize the infected cells and to destroy them by cytolytic mechanisms, but they also produce type-1 cytokines that eliminate the virus without producing tissue damage (Fig.-1).

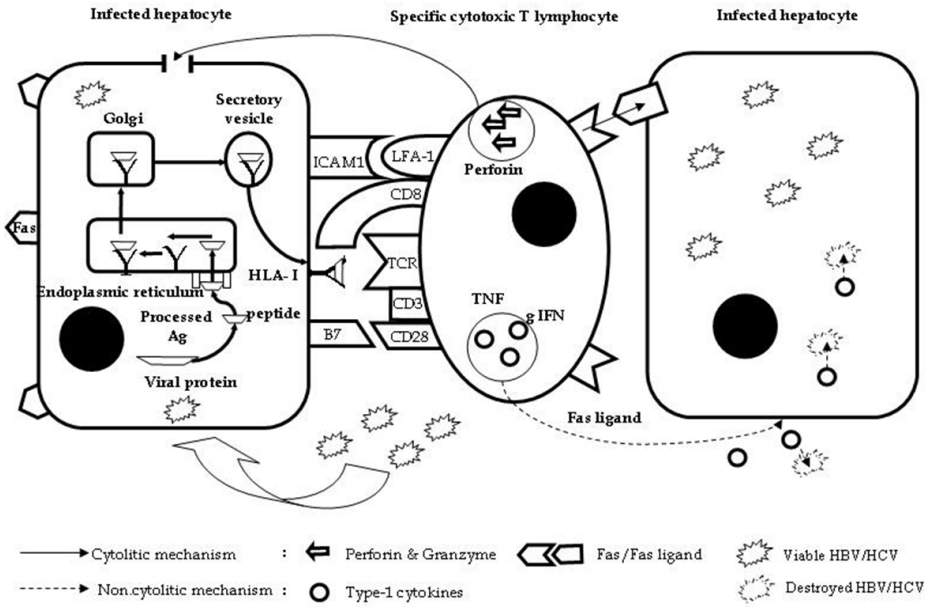

Fig. 1. Cytolitic and non-cytolitic mechanisms to destroy hepatotropic viruses by specific cytotoxic T cells

Both CD4+ and CD8+ cell activation depends on the engagement between T cell receptor and the MHC molecule/epitope complex plus the interaction between co-stimulatory molecules and their ligands (Choudhuri *et al.*, 2005). When these cells have finished their effector task, they express negative co-stimulatory molecules and pro-apoptotic factors to switch-off their activity, and a subsequent constriction in the specific T cell population is produced. After this event, a memory T cell population is maintained for decades to respond faster to a new infection, and in certain cases to keep under control viral occult infection (Appay *et al.*, 2008).
In this chapter the specific features of the immune response against two hepatotropic non-cytopathic viruses (HBV&HCV) able to induce a persistent infection in human are reviewed.

2. HBV immunopathogenesis

HBV is an enveloped incomplete circular double strand DNA virus. This virus is spread around the world and more than 2 billion people have markers of current or past HBV infection, developing chronic infection in approximately 350 million people. Approximately a quarter of persistent infection patients will develop terminal liver disease. The infection is acquired by parenteral, vertical and sexual transmission, and although there is an efficient vaccine, this infection is still an overwhelming health problem, especially in developing countries. Natural HBV control is based on a competent immune response but this is not obtained in 5-10% of infected adults and up to 95% of newborns from HBeAg-positive mothers (Liaw *et al*, 2010). Currently, there are different effective treatments able to control HBV replication but they are not very efficient in inducing either HBeAg or HB surface (HBsAg) Ag seroconversion (Perrillo *et al*, 2010). For this reason, it is interesting to understand the HBV immunopathogenesis to develop immunomodulatory strategies to restore an efficient anti-HBV immunoresponse.

2.1 Life cycle of HBV

Hepatitis B virus (HBV) is not directly cytopathic for the hepatocyte. During the early phase of HBV (before virus-specific T cells enter into the liver), there is no histological or biochemical evidence of hepatocyte damage (Guidotti *et al.*, 1999). Moreover, when cellular immune responses are deficient or pharmacologically suppressed, HBV can replicate at high levels in the liver in the absence of detectable pathological consequences (Ferrari *et al.*, 2003; Wieland *et al.*, 2000). These results suggest that hepatocyte damage during HBV infection is an immune-mediated event. Therefore, this virus is capable to enter, replicate and spread in human hepatocytes without causing any direct damage.
HBV is able to attach to the hepatocyte in a non-cell-type specific manner through cell-associated heparan sulphate proteoglycans. Later, the virus binds irreversibly to an unknown hepatocyte-specific preS1 receptor. After that, two different entry pathways have been proposed: endocytosis and fusion. Finally, the cytoplasmic release of the viral nucleocapsid, containing the relaxed circular partially double stranded DNA (rcDNA), is performed. Then, the nucleocapsid with the rcDNA is transported to the host cell nucleus (Kann *et al.*, 2007). Once rcDNA enters into the nucleus is repaired to complete the double strand DNA to produce the covalently closed circular DNA (cccDNA). The cccDNA stays stable in the hepatocyte nucleus for decades, and it is organized as chromatin like structure (minichromosome) (Levrero *et al.*, 2009). The cccDNA utilizes the cellular transcriptional machinery to produce all viral RNAs necessary for protein synthesis and viral replication.

From an immunological point of view, the cccDNA is extremely important since it will persist in most of the hepatocytes and it is not possible for the immune system to destroy it. For this reason, even if the immune response is able to control HBV infection, it does not mean HBV eradication because cccDNA persists as occult HBV infection in the hepatocytes (Larrubia, 2011; Rehermann *et al.*, 1996). From the pregenomic HBV RNA reverse transcription is performed by HBV DNA polymerase. This new HBV DNA can be either re-imported into the nucleus to form additional cccDNA molecules or can be enveloped with HBV translated proteins for secretion (Urban *et al.*, 2010).

2.2 HBV acute infection

2.2.1 Innate immune response during acute HBV infection

During HBV primo-infection, replication can be efficiently limited by type I IFNs (Wieland *et al.*, 2000; McClary *et al.*, 2000). Nevertheless, data on acutely infected chimpanzees have shown a lack of detection of genes associated to innate response in the liver during the entry and expansion phase of HBV (Wieland *et al.*, 2004). During this phase, HBV can replicate unchecked to extremely high levels. It has been proposed that, because HBV replicates within nucleocapsid particles, viral replicative intermediates of single-stranded RNA or viral DNA, which are strong activators of type I IFN genes, are protected from cellular recognition (Wieland & Chisari, 2005).

Such early events are difficult to analyze during natural infection in humans, because HBV-infected patients are mainly detected after clinical hepatitis, which occur 10-12 weeks after infection. Nevertheless, it is interesting to note that the lack of early symptoms (such as fever and malaise) in HBV-infected patients, typical of other human viral infections, constitutes an indirect evidence of the defective type-I IFN production during the early phases of HBV infection.

In a cohort of patients, sampled in the pre-clinical phase and followed up to infection resolution, serum concentrations of IFN-alpha remained barely detectable during the early incubation phase and throughout the peak of viral replication and subsequent viral load reduction. Circulating IFN-alpha levels in patients with acute HBV infection at the time of peak of viremia were no significantly greater than at the time of infection resolution. Similarly, IFN-kappa and IL-15, which are important for induction of NK effector function, were not induced during the peak of viremia (Dunn *et al.*, 2009).

Consequently, HBV can be considered as a "stealth virus", capable of sneaking through the front line of host defenses. It is possible that this situation of immune suppression might be activated by HBV replication. IL-10 is a potent immunosuppressive cytokine that can inhibit both innate and adaptive immunity. In fact, a close correlation between circulating IL-10 and HBV-DNA levels have been observed. IL-10 increased early in the course of infection, in parallel with the rapid increase in HBV viral load and antigenaemia and before the onset of inflammation. Moreover, the reduction of IL-10 coincided with either the termination of viremia or with HBeAg seroconversion. Consequently, there may be an active suppression of NK responses mediated for IL-10. In further support of this, addition of exogenous IL-10 during in-vitro experiments was able to suppress NK cell IFN-gamma production which was recovered upon blocking IL-10 and its receptor (Dunn *et al.*, 2009).

Although no induction of type-I interferon is observed, within hours after HBV infection, there is a transient release of IL-6 and other proinflammatory cytokines (IL-8, tumour necrosis factor (TNF) alfa, IL-1B). The IL-6 released was shown to control HBV gene

transcription and replication in hepatocytes shortly after infection, ensuring an early control of virus replication, thereby limiting the activation of the adaptive immune response and preventing death of the HBV-infected hepatocytes in the early phases of infection (Hosel *et al*., 2009). The production of IL-6 and other cytokines seems transient after HBV infection. Interestingly, HBV replication tends to increase 3-4 days after infection, when IL-6 level has returned to baseline. This may suggest that the virus actively counteracts the action of IL-6, like occurs during the human cytomegalovirus infection (Gealy *et al*., 2005).

However, a role for the innate immune response in the control of early HBV replication should not be dismissed. A study performed in woodchucks (Guy *et al*., 2008) observed a NK and NKT cell response within hours after inoculation with a liver-pathogenic dose of woodchuck hepatitis virus. These immune responses were at least partially capable of limiting viral propagation but were not followed by a prompt adaptive T cell response, which was delayed for 4-5weeks. Chimpanzees able to control the virus show a typical acute phase of disease with a robust activation of IFN-gamma, and TNF-alpha (Guidotti *et al*., 1999). It is possible that this initial host response to HBV is primarily sustained by NK and NKT cells, that are capable to inhibit HBV replication in-vivo (Kakimi *et al*., 2000), as shown by the early development of NK and NKT responses in healthy blood donors who became hepatitis B surface antigen and HBV DNA positive (Fisicaro *et al*., 2009). Also, an early activation of NK and NKT cells in a woodchuck model of acute hepatitis B infection has been shown. In this model NK and NKT cells induced a transient, but significant reduction of virus replication (Guy *et al*., 2008).

In human, a study performed in two seronegative blood donors who became positive for HBsAg and HBV DNA, who were monitored throughout very early stages of infection, demonstrated that the human innate immune system is indeed capable of sensing HBV early after infection and of triggering a NK/NKT cell response to contain HBV infection and to allow a timely induction of adaptive response (Fisicaro *et al*., 2009).

Therefore, rather than being silent, hepadnaviruses may be efficient at counteracting the actions of the innate immune system early after infection. There is a growing body of evidence suggesting that HBV could inhibit innate responses by regulating the expression of Toll-like receptors (TLRs), which are major sensors of viral infection in immune-specialized and non-specialized cells (Barton, 2007). HBV is able to suppress toll-like receptor-mediated innate immune response in murine parenchymal and non-parenchymal liver cells (Wu *et al*., 2009). Indeed, the expression of TLR1, TLR2, TLR4 and TLR6 is significantly lower in peripheral blood mononuclear cells (PBMC) and hepatocytes from chronic hepatitis B (CHB) patients (Chen *et al*., 2008). Furthermore, flow cytometric analysis has shown that the expression of TLR2 in PBMC, from CHB patients is significantly decreased. TLR2 expression on PBMC has been correlated with the HBsAg plasma levels (Riordan *et al*., 2006) and HBeAg protein (Visvanathan *et al*., 2007). Recently, an immunomodulatory role of HBeAg on innate immune signal transduction pathways, via interaction and targeting of TLR-mediated signalling pathways, has also been shown (Lang *et al*,.2011).

Moreover, dendritic cells (DC) exhibit functional impairment in hepatitis B virus carriers. Plasmocytoid (p)-DC are the major type-I interferon producing cells and sensors of viral infections because they express both TLR7 and TLR9 that respectively recognize, even in absence of viral replication, single-stranded RNA and unmethylated cytosine-guanosine dinucleotide motifs (Fitzgerald-Bocarsly *et al*., 2008). A recent study reported that, in CHB patients, there was a reduction of TLR-9 expressions in pCDs, which correlates with an impaired IFN-alpha production by these cells (Xie *et al*., 2009).

Altogether, these data suggest that HBV infection can alter innate immune responses, triggered by both specialized cells and hepatocytes, through down-regulating functional expression of TLR. Currently, whether HBV is a stealth virus for the innate immune response or is able to block it efficiently is a matter of debate.

2.2.2 Adaptive response during acute HBV infection

Despite of the lack of proper innate response activation, this does not affect to adaptive response during HBV primo-infection. HBV-specific T cell response appears soon after the exponential HBV replication phase (Webster *et al.*, 2000). Both, CD4+ and CD8+ specific responses are present and they are polyclonal, vigorous and multi-specific, when the viral control is obtained, while these responses are impaired when the infection progresses over chronicity (Maini *et al*., 1999). HBV control is achieved through the labor of HBV-specific CD8+ T cells. These cells are able to recognize infected hepatocytes and to destroy them by apoptosis, but also they produce type-I cytokines, such as gamma-interferon and TNF-alpha, which are capable of non-cytopathic HBV clearing (Ferrari *et al.*, 2003; Guidotti & Chisari, 2001). This response to become fully activated needs the adequate stimulation by professional antigen presenting cells and the correct regulation by specific CD4+ cells. HBV-specific CD8+ T cells are responsible of HBV control, but they also initiate a minor liver damage. In fact, most HBV DNA is eliminated by non-cytolitic pathways before amino-transferases elevation is detected. Nevertheless, the secreted IFN-gamma by these cells, in addition to the chemokines produced by infected hepatocytes, attracts non-specific mononuclear cells and polymorphonuclear cells, which are responsible of liver damage amplification (Guidotti & Chisari, 2006). This phenomenon is also acting in the pathogenesis of chronic disease. Specifically, during persistent infection, the HBV specific response is impaired and unable to control the infection, but the hepatocytes continue secreting chemokines to attract effector T cells. However, non-specific inflammatory cells are also attracted and they are the cause of the low grade of persistent liver damage (Bertoletti & Maini, 2000).

During the acute phase of infection, antibodies (Ab) against HBsAg, HBeAg and core (HBc) Ag are produced by activated B cells. HBsAb and HBeAb production is T helper dependent, while HBcAb secretion is dependent and non-dependent from T helper action (Milich & Chisari, 1982). HBs antibodies are produced very early after infection, but they are not detected because they generate complexes with circulating antigens, and therefore they are not detected until the virus is controlled. HBs antibodies prevent viral spreading from one to another hepatocyte and also block circulating HBV. The detection of these antibodies means HBV control and confers natural immunity against re-infection. Observation of HBsAb occurs when HBV is controlled by immune system, and these are neutralizing Abs that will avoid HBV re-infection in case of a new encounter with the virus. HBc Abs are not neutralizing and they indicate HBV contact. When HBc IgM subtype is positive it means acute infection or HBV flare-up during chronic infection. HBe Abs appear before HBs Abs during acute HBV recovery and also when chronic patients shift from a replicative to a non-replicative phase. Moreover, HBe Abs are also present during the HBV chronic replicative phase, when the infecting virus displays a pre-core mutation that avoids HBe Ag production (Maruyama *et al*., 1994; Milich & Liang, 2003).

During adulthood, most of acute HBV infected cases recover and develop natural immunity due to the combination of a polyclonal, vigorous and multispecific cytotoxic and helper

response (Guidotti & Chisari, 2006). After a self-limited infection, a T cell response constriction is observed and a central memory T cell population is generated. In these cases, a long-lasting protective T cell response is developed. These cells keep under control the intrahepatic HBV traces for decades. In fact, in HBV recovered patients it is possible to demonstrate a T1 orientated multispecific cytotoxic and helper response, decades after primo-infection, and those responses are associated with the observation of HBV DNA in sera or PBMC using ultra-sensitive PCR techniques. These data show that HBV recovery does not mean HBV eradication, since despite of clinical recovery it is possible to demonstrate HBV viral traces that are maintained under control due to the adaptive memory immune response (Larrubia, 2011; Penna *et al.*, 1996; Rehermann *et al.*, 1996).

2.3 HBV chronic infection

Around 5-10% of HBV primoinfection progresses to chronicity in adult infection, while it reaches 95% of newborns from HBeAg-positive mothers and approximately 50% during childhood infection (Liaw *et al.*, 2010). The development of a persistent HBV infection is based on a failure of HBV-specific response due to the induction of an anergic and pro-apoptotic status on this response because of the high viral pressure (Maini *et al.*, 2000a; Webster *et al.*, 2004). Several mechanisms have been involved in the impairment of specific T cell response. Specific T cells behave as anergic cells with progressive impairment of type-1 cytokine production, such as IL-2, IFN-gamma and TNF-alpha. The cytotoxic T cells are neither able to proliferate nor to kill infected hepatocytes after antigen encounter. Nevertheless, cytokines and chemokines produced in the infected liver are able to attract a non-specific inflammatory population causing the persistent liver damage. Several mechanisms are used by HBV to induce this anergic status, which will end-up in a pro-apoptotic situation that could cause specific T cell deletion. Persistent high HBs antigenemia, massive production of defective viral particles and the toleraising liver environment induces an anergic condition on T cells. In fact, HBV infected liver is depleted in tryptophan and there is an accumulation in its toxic metabolite (IDO) which is able to induce immunotolerance (Larrea *et al.*, 2007). Also, arginase I activity is increased during HBV infection provoking an arginine depletion on T cells which causes a CD3ζ down-regulation. The effect of CD3ζ lower expression translates into IL-2 production impairment by HBV-specific CD8+ cells (Das *et al.*, 2008). Interestingly, in the HBV infected liver is increased the secretion of immunosuppressive cytokines. IL-10 is produced by dendritic cells and Kupffer cells while transforming growth factor-beta (TGF-β) is secreted by stellate cells. The level of these cytokines correlates with HBV disease activity during chronic and acute infection (Dunn *et al.*, 2009). Other escape mechanisms involve TRAIL-mediated deletion of HBV-specific CD8+ cells by NK cells (Dunn *et al.*, 2007). Moreover, regulatory T cells can cause HBV-specific T cell activity suppression (Furuichi *et al.*, 2005). On the other hand, persistent HBV infection favors the up-regulation of pro-apoptotic molecule Bim. This molecule mediates premature HBV specific cytotoxic T cell death following intrahepatic antigen presentation (Lopes *et al.*, 2008). Another common mechanism, induced by HBV to evade immune system, is the induction of negative co-stimulatory molecules such as CTLA-4, PD-1, Tim3 and Lag3. Excessive co-inhibitory signals drive T cell exhaustion during chronic HBV-infection (Maini & Schurich, 2010). Finally, HBV is also able to evade specific immune response by developing escape mutation at cytotoxic and helper immunodominant epitopes (Maini *et al.*, 2000b).

2.3.1 Adaptive response during chronic HBV infection

Chronic evolving infection is characterized by several progressive phases with different adaptive response features. The first stage is called immunotolerant phase. This is typical for countries with high rates of mother to child HBV transmission, but it is not seen in Western countries, where this route of transmission is not common. During this phase, HBV viral load is extremely high, but the liver damage and the anti-HBV immune response are absent. Several studies from D. Milich group, in HBe+ transgenic mice, have shown that the lack of HBV-specific immune response is due to some properties of HBeAg. This viral protein is able to cross the placenta to reach the offsprings thymus, where this is considered a self-antigen, eliciting HBe/HBc Ag-specific T helper cell tolerance in uterus (Milich *et al.*, 1990). Moreover, during this phase, high HBV viral load inhibits adaptive immune response. In fact, frequency and function of HBV-specific T cells is inversely correlated with HBV viral load (Boni *et al.*, 2007; Webster *et al.*, 2004) (Fig.-2). In the natural history of chronic HBV infection, this phase is followed by the immuno-clearance stage. This is the common starting point in persistent infection in Western countries. This phase is characterized by viral replication and liver damage fluctuations. Even though the specific immune response is quite inefficient, it is still able to obtain certain HBV control. During this phase, HBeAg seroconversion and HBV pre-core mutant selection is possible. HBe seroconversion allows the change to another HBV infection phase with a higher viral control and lower liver damage. HBe seroconversion is faster in individuals with certain polymorphisms at IL-10 and IL-12 genes. In these cases, high levels of IL-10 and IL-12 are observed and they are a predictor of spontaneous HBe seroconversion (Wu *et al.*, 2010). Another typical feature of the immuno-clearance phase is the presence of HBV exacerbations, characterized by HBeAg level increase followed by transaminase level raise. The HBeAg level increase induces an activation of HBc/HBe specific response activation, after this a decrease in HBeAg and transminase level is observed, followed by a specific T cell response constriction. This data show that HBV-specific T cell activation due to HBeAg level is causing acute exacerbations in HBeAg+ chronic patients (Frelin *et al.*, 2009). This phenomenon can lead to liver damage generation, HBe seroconversion and pre-core mutant selection. During these HBV acute exacerbations, HBV-specific cytotoxic T cells destroy wild-type HBV infected hepatocyte producing liver damage. Moreover, if along this stage HBV pre-core mutants emerge, these cytotoxic T cells can select them, since the infected hepatocytes with these variants are not recognized properly by cytotoxic T cells. In fact, liver infected cells by the wild type virus are eliminated more efficiently by specific cytotoxic T cells than cells infected by the pre-core mutant. This is because wild-type infected cells presenting HBc and HBe epitopes are better targets for cytotoxic T cells than cells infected by HBV pre-core mutant expressing only core epitopes (Frelin *et al.*, 2009). This situation leads to HBe antigen negative form of chronic hepatitis B with persistent liver damage, which is different to the wild-type HBe seroconversion where the infection can be consider inactive. This last one is the third phase of the chronic HBV natural history which is called low or non replicative phase, and corresponds to the clinical inactive carrier state. In this stage viral load and liver damage is very low. During this phase HBV-specific T cell responses are present and are quite efficient despite lack of liver damage. These cells are very competent in controlling infected hepatocytes, preventing HBV spreading and the development of liver infiltration by non-specific inflammatory cells, which are the cause of persistent liver

damage during chronic active hepatitis B. Therefore, it is considered that during the low/non-replicative phase HBV is under a partial control by HBV-specific response (Maini *et al.*, 2000a). At this stage, it is possible to observe HBV reactivation associated with hepatitis flares, mainly in the case of infection by HBV pre-core mutants. This last phase of HBV natural history is called reactivation phase. The immunological causes of these reactivations are not very well known yet. During these hepatitis flares is not possible to demonstrate the presence of HBV-specific T cell reactivity, but it is observed NK cell activation which correlates with the degree of liver damage (Dunn *et al.*, 2007). Therefore, in this last step of chronic HBV natural history, the innate response could be involved.

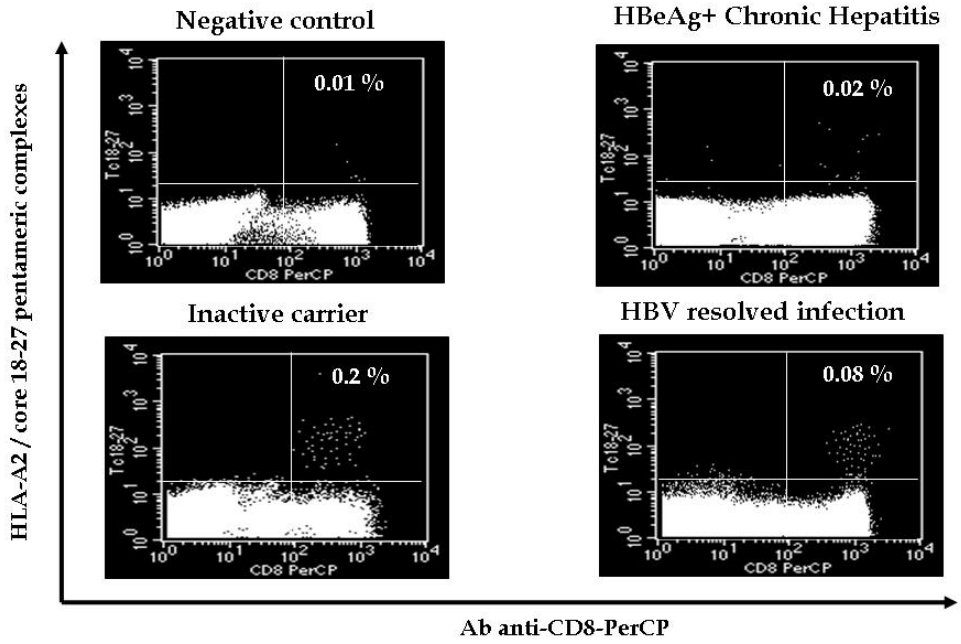

Fig. 2. FACS® dot-plots from peripheral blood mononuclear cells of HBV infected patients with different HBV control stained directly ex-vivo with Ab against CD8 plus multimeric HLA-A2/core 18-27 complexes. A negative correlation between viral control and frequency of HBV-sepcific CD8+ cells is observed. Figures in the upper right quadrant show the frequency of HBV-specific CD8+ cells out of total CD8 population.

In summary, HBV is not ever completely eliminated from the infected host, but there is a gradient of control according to the functional efficiency of HBV-specific response. In patients with HBV natural immunity, they present a HBV occult infection with a very efficient control by CD4+ and CD8+ specific HBV T cells. This immune control is partial in patients in the inactive carrier state and completely inefficient in cases with chronic active hepatitis (Boni *et al.*, 2007; Maini *et al.*, 2000a; Zerbini *et al.*, 2008). Strategies directed to restore anti-HBV adaptive response could help in the permanent infection control.

3. HCV immunopathogenesis

The hepatitis C virus (HCV) is an enveloped; positive stranded RNA virus and represents the Hepacivirus genus in the Flaviviridae family. It has been estimated that more than 170 million people are infected with HCV, since clinical identification and molecular cloning of HCV in late 1980s. This virus is spread by contact with infected blood and body fluids. Approximately 80% of infections succeed in establishing a chronic infection with the potential for developing severe liver diseases such as cirrhosis and hepatocellular carcinoma (HCC) (Lavanchy, 2009; Tsukuma *et al*., 1993).

No effective vaccine against HCV is available till date. Current standard-of-care therapy for HCV infection as peg-interferon-alpha and ribavirin (Pawlotsky, 2004), has limited efficacy, in particular against the genotype 1 virus (Fried *et al*., 2002; Manns *et al*., 2001). An extended search for new therapy is progressing, already passed for marketing authorization of the protease-inhibitors (Poordad *et al,* 2011). A major concern with new therapy is rapid development of drug-resistant viral mutants. Due to the failure or side effect of the treatment, stepping forward for understanding the immunopathogenesis of HCV infection is essential in the development of a therapeutic vaccine and immunomodulatory treatments for chronic infections.

Due to the lack of adequate cell culture systems, HCV studies have been slowed down for a long time, but continuous progress in the last few years it has overcome this obstacle. In-vivo model to study the biology of HCV have been significantly restricted due to the limited experimental availability of chimapanzees, the primary model for HCV (Alter *et al*., 1978; Bukh, 2004), and difficulties encountered in reproducing true infection in small animals. Two breakthroughs has been an important contribution to the field: firstly, subgenomic replicons (i.e. without structural genes) (Blight *et al*., 2000; Blight *et al*., 2003; Lohmann *et al*., 1999), which are highly permissive for HCV replication (Blight *et al*., 2002) and secondly, HCV complete replication in cell culture (Lindenbach *et al*., 2005; Wakita *et al*., 2005; Zhong *et al*., 2005). However, it has long been recognized that these models are complicated by the particularly high error rate of the HCV RNA replicase (Rong *et al*., 2010).

It is widely accepted that immune-mediated host-virus interactions are responsible for the outcome of HCV and pathogenesis of further severe diseases. In this chapter, it is covered how virus evades primary defense mechanisms. Finally, adaptive immune response escape mechanisms induced by HCV to become persistent are also analyzed. To be familiar with pathogenesis of HCV infection, a brief outline of HCV life cycle is provided below.

3.1 Life cycle of HCV

The development of HCV replicons (Blight *et al*., 2000; Blight *et al*., 2003; Ikeda *et al*., 2002; Lohmann *et al*., 1999), HCV pseudotyped particles (HCVpp) (Bartosch *et al*., 2003a) and most recently the infectious HCV cell culture system (Lindenbach *et al*., 2005; Wakita *et al*., 2005; Zhong *et al*., 2005) have advanced our understanding of the viral life cycle. Hepatocytes are the primary site of HCV infections. HCV life cycle begins with binding of the virus to cell surface receptors. The putative receptors, the tetraspanin protein CD81 (Bartosch *et al*., 2003a; Hsu *et al*., 2003; Pileri *et al*., 1998; Wunschmann *et al*., 2000), the scavenger receptor class B member I (SR-B1) (Bartosch *et al*., 2003a; Grove *et al*., 2007; Kapadia *et al*., 2007; Scarselli *et al*., 2002) and the tight junction proteins claudin-1 (Evans *et al*., 2007) and occluding, (Benedicto *et al*., 2009; Liu *et al*., 2009; Ploss *et al*., 2009) have all been shown to enable HCV entry. In addition, the low-density lipoprotein receptor (Agnello *et al*., 1999;

Molina *et al.*, 2007; Monazahian *et al.*, 1999; Wunschmann *et al.*, 2000), asialoglycoprotein receptor (Saunier *et al.*, 2003), and glycosaminoglycans (heparin sulfate) are also involved, but their exact roles have not been determined. By clathrinmediated endocytosis (Blanchard *et al.*, 2006; Meertens *et al.*, 2006), HCV enters the cell. The virus undergoes an uncoating process by fusion between the viral envelope and endosomal membrane in the acidified endosomal compartment (Bartosch *et al.*, 2003b; Haid *et al.*, 2009; Hsu *et al.*, 2003; Koutsoudakis *et al.*, 2006; Lavillette *et al.*, 2006; Tscherne *et al.*, 2006) via E1/E2-mediated class II fusion (Garry & Dash, 2003; Lavillette *et al.*, 2007), to expose the viral genomic RNA to host-cell machinery. About ~9.6 kb viral RNA genome is released into the host cell cytoplasm, to serve as template for the translation of the viral proteins. IRES-mediated translation of the HCV genome produces a single ~3,000 amino-acid polyprotein (Moradpour *et al.*, 2004), which is processed by cellular and viral proteases into at least 10 different protein products. These products include the structural proteins, which form the viral particle (the virus core and the envelope proteins E1 and E2), and the nonstructural proteins P7, NS3, NS4A, NS4B, NS5A and NS5B (Guidotti & Chisari, 2006). Viral replication is driven by minus strand intermediate. HCV double stranded RNA (dsRNA) is freely exposed in the cytoplasm of infected cell (Moradpour *et al.*, 2004), which is recognizable for host innate immune system. Nucleocapsid is formed by assembling capsid proteins and genomic RNA and bud through intracellular membranes into cytoplasmic vesicles. Finally, by secretory pathway, mature enveloped virions release from the cell.

3.2 Innate immune response during acute HCV infection

The first response to HCV protein is thought to be IFN-β production by infected hepatocytes, which are able to secrete type I IFN. The infected cells are sensed with pathogen associated molecular patterns (PAMP), Toll like receptor (TLR3) (Marie *et al.*, 1998) and retinoic acid–inducible gene I (RIG-I) (Bauer *et al.*, 2001; Sato *et al.*, 2000) by endosomal dsRNA and cytosolic dsRNA respectively, which is an essential intermediate in the HCV replication cycle, and thus, they may be important in the pathogenesis of hepatitis C (Saito *et al.*, 2008). RIG-I recruits IFN-β promoter stimulator protein 1 (IPS-1; also called CARD adaptor inducing IFN-β CARDIF), virus-induced signaling adapter (VISA), and mitochondrial antiviral signaling protein (MAVS) (Hoshino *et al.*, 2006; Meylan *et al.*, 2005; Xu *et al.*, 2005), after ATP-driven activity dependant on recognition of viral protein (Honda *et al.*, 2004). On other hand, TLR3 dimerization, due to leucine-rich repeats (Liu *et al.*, 1998), recruits the adapter protein, Toll–IL-1 receptor domain–containing adaptor inducing IFN-β (TRIF). Both processes result in downstream signaling, nuclear translocation of IFN regulatory factor 3 (IRF3) and leads to stimulation of the transcription of a set of genes including IFN-β (Kawai & Akira, 2008). Antiviral state, induced by secreted IFN β, gives an alert to uninfected cells by activation of effector molecules. Binding of IFN α-β to cognate receptor complex lead to the activation of JAK/STAT pathway, which results in the induction of IFN-stimulated genes (ISGs) and lead to enhance the IFN response (Rehermann, 2009) (Fig.- 3).

However, HCV has organized a number of countermeasures not only to inhibit the induction phase, but also interfere with the effector phase of the IFN system (Fig.- 3). It has been confirmed, in in-vitro studies, that HCV serine protease, NS3/4A is enable to cleave MAVS (Li *et al.*, 2005b), TRIF (Li *et al.*, 2005a), IPS-1 (Foy *et al.*, 2003) and oligomerization of MAVS, which is part of signaling process (Kawaguchi *et al.*, 2004; Li *et al.*, 2005a; Li *et al.*, 2005b; Marie *et al.*, 1998; Meylan *et al.*, 2005; Sakamoto *et al.*, 2000). Disruption of IRF-3

activation occurred by NS3 protein action (Liu *et al*., 1999) and it has been shown with different cell lines in-vitro studies (Kawaguchi *et al*., 2004; Marie *et al*., 1998). Another key player, HCV core, when over expressed in cell culture, disturbs antiviral activity via interfering in JAK/STAT signaling and ISG expression by inhibition of STAT1 activation. Simultaneously it induces its degradation (Gale & Foy, 2005; Lin *et al*., 2006) by induction of inhibitor of the JAK/STAT pathway SOCS3 (Bode *et al*., 2003), protein phosphatase 2A (PP2A), which ultimately reduces the transcriptional activity of ISG factor 3 (ISGF3) (Heim *et al*., 1999); and inhibition of ISGF3 interaction to IFN-stimulated response elements (Rehermann, 2009). HCV NS5A interferes with the function of ISGs by inhibiting 2′-5′ oligoadenylate synthetase (2′-5′ OAS) and leads to overall ISG expression impairment (Polyak *et al*., 2001). Protein kinase R (PKR) can negatively regulate HCV replication noncytolytically in cell cultures (Kim *et al*., 2004; Zhao *et al*., 2004), which can interacts with HCV NS5A and lost its function. Interestingly, HCV E2 acts as distraction target to PKR (Taylor *et al*., 1999). To sum up, the main targets of HCV proteins to evade immune response are interference with the induction of IFN synthesis, IFN- induced intracellular signaling and IFN-induced effector mechanisms (Fig.-3).

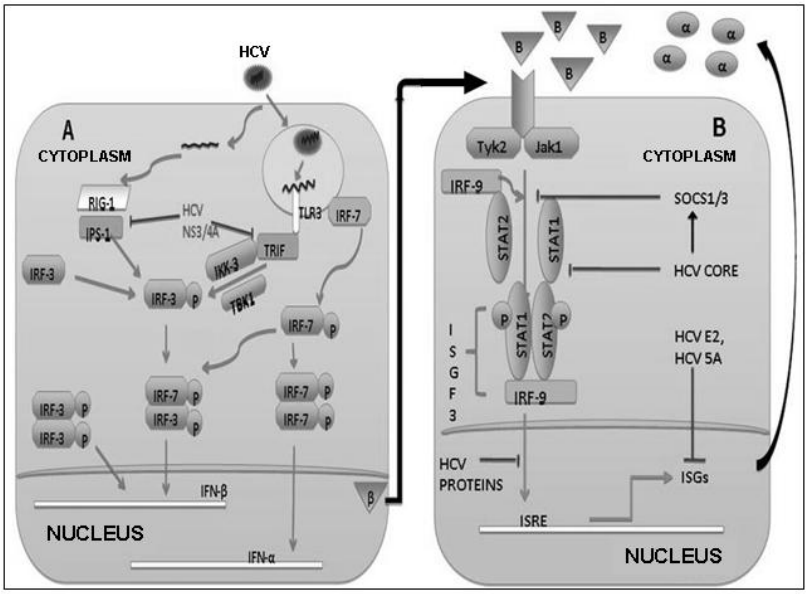

Fig. 3. Evasion of Innate immune response by HCV: (A) Interference in IFN synthesis: Blocking of TLR 3 and RIG-1 signalling respectively, by cleavage of the adaptor molecule TRIF and IPS-1 via HCV NS3/4A; (B) Interference in IFN-induced effector mechanisms: Binding of IFN β and its receptor with TYK2 and JAK1 kinase activation lead to form ISGF3 complex, where this complex interact with IFN stimulated response elements (ISREs) within the promoter and enhancer region of ISGs to induce ISGs (such as 2′, 5′ OAS, PKR, IRF7) production in nucleus. HCV core induce SOCS1/3, which is the inhibitor of the JAK/STAT pathway and inhibits STAT1 phosphorylation, which inhibits assembly of trimeric ISGFs complex. Function of ISGs is inhibited by HCVE2 and HCV NS3/4A.

Dendritic cells (DC) are professional antigen presenting cells with important functions in antiviral immunity through activation of adaptive immune responses. Type-I IFNs are also produced by pDCs, which derive from the lymphoid lineage. Although, production of IFN alpha/beta, in early phase of infection occurs after recognition of ssRNA and dsRNA by TLR7 and TLR9 respectively, the mechanism is still not clear (Albert *et al.*, 2008). The frequency of pDCs in the blood (Nakamoto *et al.*, 2008) and their production of IFN-α in HCV infection is reduced after in vitro stimulation (Bowen *et al.*, 2008). The possible mechanism has been demonstrated in in-vitro studies. First, HCV core and NS3 activate monocytes by TLR2 signaling to produce TNF-α (Izaguirre *et al.*, 2003), which in turn inhibits IFN-α production and induces pDC apoptosis (Bowen *et al.*, 2008). Second, HCV itself inhibits IFN-α production of pDCs (Diepolder *et al.*, 1995). However, other studies revealed regular response to TLR stimulation by circulating pDCs of chronically infected individuals (Decalf *et al.*, 2007; Longman *et al.*, 2005) and they have high levels of endogenous type I IFNs without immuno-dysfuction (Albert *et al.*, 2008). Although this defense mechanism is significant, the host rarely overcomes HCV infection, which suggests several other viral evasion mechanisms that are poorly or not understood yet.

Another group of DCs, myeloid DCs (mDCs) derive from the myeloid lineage (Lanzavecchia & Sallusto, 2001; Steinman *et al.*, 2003). Due to its tolerogenic and stimulatory role (Lanzavecchia & Sallusto, 2001; Steinman *et al.*, 2003), mDCs have been broadly studied in HCV infection. mDCs have not been observed to be decrease in peripheral blood or dysfunctional in HCV chronic infected individuals in in-vitro studies (Kanto *et al.*, 1999; Longman *et al.*, 2004). Nevertheless, HCV proteins can interact with monocytes/macrophages through TLR2, inducing the IL-10 production, which hampers IL-12 production by mDC and IFN-alpha by pDC, or they directly inhibit DC differentiation (Szabo & Dolganiuc, 2005). IL-12 cytokine production by mDC is decreased in HCV patients in response to stimuli like CD40 L or poly (I:C) (Anthony *et al.*, 2004), which can explain clearly the shift from Th1 to Th2 response in HCV patients. In-vitro studies indicates that DC expressing core and E1 proteins have lower stimulatory ability, which is associated to the lack of maturation after stimulation with TNF-alpha or CD40L (Sarobe *et al.*, 2003).

Other cells involved in the innate response are the NK cells. Functions of these cells include generating a cytotoxic response, regulatory cytokines production and control on DC maturation and amplitude of DC response, which may deeply impact on type of down-stream adaptive immune responses. Response to HCV infection by NK cell is direct apoptosis induction of infected cells with production of antiviral cytokines (Golden-Mason & Rosen, 2006; Lodoen & Lanier, 2006). Moreover, NK cell depletion or dysfunction favor HCV persistence (Golden-Mason *et al.*, 2008). The role of interactions between HLA class I and killer cell–Ig-like receptors (KIR) during HCV infection has been shown. KIR can regulate NK cell activities. However puzzling contradictions for this topic in different studies have been revealed (Montes-Cano *et al.*, 2005; Paladino *et al.*, 2007; Rauch *et al.*, 2007). The importance of NK cells in the resolution of HCV infection is illustrated by the influence of genetic polymorphisms of KIR and their HLA ligands on the outcome of HCV infection, which was dependent on a homozygous HLA class I ligand background (Khakoo *et al.*, 2004; Knapp *et al.*, 2010; Stegmann *et al.*, 2010). There is need to focus on clear understanding of functional and molecular HLA-KIR interactions to know about the possible way for NK cell-mediated protection in animal models of HCV infection.

However, an increased proportion of NK cells expressing activating receptors, enhanced cytotoxicity and defective cytokine production have revealed in chronic HCV infection (Oliviero *et al.*, 2009). Megan et al revealed that IL28A cytokine could significantly inhibit IFN-γ production lead to NK cell inactivation (Ahlenstiel *et al.*, 2010), which would be important to attenuate chronically activated NK cells. Consequently, the analysis of functional scene between NK cells and type 3 IFN in the immune response to virus will be required to understand the role of the NK in disease progression during HCV infection.

3.3 Adaptive immune response

The second barrier to control HCV infection is the adaptive immunity. This response has two arms to fight against pathogens; humoral and cellular immune response. Humoral immune response, that means neutralizing and non-neutralizing antibodies can endorse antiviral activity and pathogenesis (Guidotti & Chisari, 2006). Cellular immune response shows antiviral immunity by means of virus specific CD8 cytotoxic T lymphocytes (CTLs) and CD4 T helper cells, which play key effector and regulatory roles respectively. These T cells take part in viral pathogenesis of HCV by direct killing of infected cells or producing soluble factors able to clear the virus in a non-cytolytic manner, but also can lead to HCV pathogenic events, favoring direct liver damage and attracting non-specific inflammatory cells to perpetuate the liver inflammation (Guidotti & Chisari, 2006).

3.3.1 Humoral immune response

Neutralizing antibodies (nAbs) generally play a critical role for controlling initial viremia and protecting from re-infection in viral infections. However, the role of the humoral immune response in the clearance of HCV infection has been in the dark for a long time due to difficulties to determine relative role of antibodies to neutralize HCV. It can exclusively be evaluated by relevant model systems. It is thought that HCV clearance could occur in the absence of nAbs. If they are present alone, these Abs are inadequate to eradicate HCV in most of the cases in early studies (Dustin & Rice, 2007; Lechner *et al.*, 2000a; Lechner *et al.*, 2000b; Logvinoff *et al.*, 2004; Thimme *et al.*, 2002).

It has been proved that HCV specific T cells may compensate for lack of neutralizing antibodies to obtain HCV clearance (Semmo *et al.*, 2006). However, due to the development of novel model systems (Bartosch *et al.*, 2003a; Baumert *et al.*, 1998; Lindenbach *et al.*, 2005; Wakita *et al.*, 2005; Zhong *et al.*, 2005), it is possible to focus on HCV entry into host cells and neutralization process which demonstrated that nAbs are induced by patients who subsequently control (Lavillette *et al.*, 2005) or resolve (Pestka *et al.*, 2007) viral infection in the early phase of infection and contrary in chronic infection. This suggests that a strong, early, broad nAbs response may contribute to resolution of HCV in the acute phase of infection while delayed induction of nAbs may contribute to development of chronic HCV infection.

Instead of the rapid, vigorous and multi-specific antiviral host immune responses, chronic patients have been shown to develop a delayed and inefficient neutralizing antibody response (Pestka *et al.*, 2007) due to HCV escape mechanism (Zeisel *et al.*, 2008). Recent studies evident that entry of HCV can be hampered or modulated by nAbs of chronic HCV patients (Gal-Tanamy *et al.*, 2008; Haberstroh *et al.*, 2008; Keck *et al.*, 2009), while it is controversial in cell culture study (Grove *et al.*, 2008). In addition, although nAbs are incapable to clear the virus in chronic infection, due to selection pressure exerting on viral

variants, they contribute to the evolution of the HCV envelope sequences to escape (Farci *et al.*, 2000; von Hahn *et al.*, 2007). It has been proposed that HCV stimulates B cells in a B cell receptor-independent manner in chronic infection (Racanelli *et al.*, 2006) and may favor the development of lymphoproliferative and autoimmune diseases (Guidotti & Chisari, 2006).
Although, in vitro studies evident that the neutralization ability of HCV-specific nAbs enhanced by complement activation against pseudotyped viruses (Racanelli *et al.*, 2006), there is absence of direct experimental evidence about the presence of any of these Ab-mediated functions during natural HCV infection. However, immune complexes are believed to play a pathogenetic role in the development of manifestations such as cryoglobulinemia, glomerulonephritis, porphyria cutanea tarda, and necrotizing cutaneous vasculitis during chronic HCV infection (Agnello & De Rosa, 2004; Amarapurkar & Amarapurkar, 2002; Manns & Rambusch, 1999).

3.3.2 Cellular immune response

Cytotoxic T lymphocyte (CTL) responses are essential to control HCV infection. Efficiency of antiviral CTL responses depends on where these cells are primed. Efficient antiviral CTL response is observed when it is primed in lymphoid organs, whereas within the liver, priming is more tend to induce T cell inactivation, tolerance or apoptosis (Guidotti & Chisari, 2006). A strong, multispecific and long-lasting T-cell immune response emerge to be important for control of viral infection (Dustin & Rice, 2007; Zhang *et al.*, 2009). Persistent HCV unsuccessfully control by T effector cells is due to multiple causes, such as: HCV escape mutant generation, immunosuppressive effects exertion, Tregs induction, or effector T cell exhaustion or apoptosis (Bassett *et al.*, 1999; Thimme *et al.*, 2006; Thimme *et al.*, 2001; Larrubia *et al.*, 2011).

3.3.2.1 Adaptive cellular response during acute HCV infection

Vigorous CD4+ and CD8+ T cell responses targeting multiple HCV regions with intrahepatic production of IFN-γ emerged in acute hepatitis C infection (Bowen & Walker, 2005; Lechner *et al.*, 2000b; Thimme *et al.*, 2001). Decreasing viral titer correlates precisely with the appearance of HCV-specific T cells and IFN-γ expression in the liver (Shin *et al.*, 2006). The appearance of HCV-specific T cells can be detectable in the peripheral blood or in the liver compartment several weeks after infection in humans or experimental chimpanzee models (Dustin & Rice, 2007; Rehermann, 2009), respective with primary peak of transaminases and irrespective of clinical outcome (resolution or chronicity). Delayed emerging of antigen-specific responses are also essential for the HCV control (Rehermann, 2009).
The protective function of CD4+ T cells appear to be due to the production of antiviral cytokines, but also their helping nature to antiviral B cells and in maintenance of CD8+ T cell memory. The HCV clearance has been observed and correlated with vigorous proliferation of specific CD4+ T cells (Diepolder *et al.*, 1995; Missale *et al.*, 1996) with concurrent IL-2 and IFN-γ production (Kaplan *et al.*, 2007; Urbani *et al.*, 2006a). The early sustained development of CD4+ T cell response needs to be successful for viral clearance (Urbani *et al.*, 2006a). Whereas, HCV-specific CD4+ T cell responses are not observed in chronic HCV infection. Moreover, the recurrent viremia has been correlated with loss of previous strong CD4+ T cell responses after several months of viral clearance (Gerlach *et al.*, 1999; Nascimbeni *et al.*, 2003). Studies on the relative importance of CD4 help in spontaneous recovery in acute HCV infection demonstrated that fact (Smyk-Pearson *et al.*, 2008). CTL priming in presence of CD4 help is critical factor in protective function (Urbani *et al.*, 2006a).

On the other hand, the magnitude of CD8+ T cells response in acute HCV infection does not correlate with the clinical or viral outcome (Francavilla *et al.*, 2004; Kaplan *et al.*, 2007; Urbani *et al.*, 2006a). Expression of a dysfunctional phenotype with weak proliferation, low IFN-γ production, impaired cytotoxicity and increased levels of the well known exhausted phenotype programmed death-1 receptor (PD-1) are found in HCV infection, irrespective of infection progression (Bowen *et al.*, 2008; Keir *et al.*, 2007; Nakamoto *et al.*, 2008; Sharpe *et al.*, 2007; Trautmann *et al.*, 2006; Urbani *et al.*, 2006b; Larrubia *et al.*, 2011). Antigen-dependent reactivity of HCV-specific CD8+ T cells has been proved by a rapid decay of CD8+ T cell responses during antiviral therapy (Rahman *et al.*, 2004). However, the appearance of self-sustaining memory T cells (CD127+ memory HCV-specific CD8+ T cells and CD4+ T cells) are necessary to control HCV infection (Lechner *et al.*, 2000b; Thimme *et al.*, 2001; Urbani *et al.*, 2006a). In fact, years after HCV control due to anti-HCV treatment it is possible to find HCV traces in association with HCV-specific T cell reactivity. These data suggest that HCV-specific memory T cells are essential to clear HCV infection completely after the initial acute clearing (Veerapu *et al.*, 2011).

3.3.2.2 Adaptive cellular response during chronic HCV infection

Complete resolved HCV patients exhibit broader CTL responses with higher functional avidity and wider cross-recognition ability than patients with persistent HCV infection (Yerly *et al.*, 2008). There are evidences that demonstrate rapid mutation in HCV genome, T cell exhaustion because of expression of inhibitory molecules (Fig.-4), immune regulatory cytokine induction and immune modulatory T reg cell activation, which are main reasons for HCV persistence in chronically infected patients (Hiroishi *et al.*2010; Pavio & Lai, 2003; Seifert *et al.*, 2004; Tester *et al.*, 2005; Larrubia *et al.*, 2011).

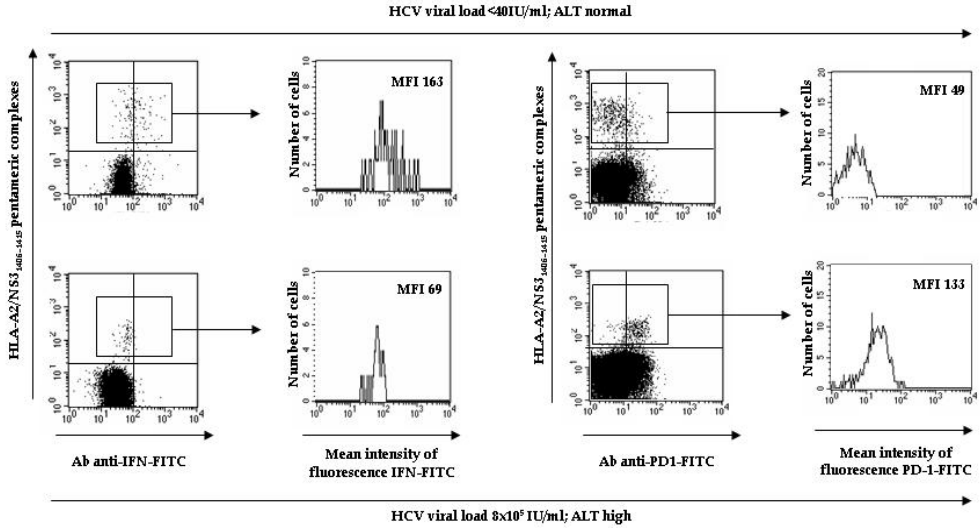

Fig. 4. FACS® dot-plots and histogramas of CD8+ cells from HCV patients with different viral control. CD8+ cells were stained with Abs anti IFN-gamma and anti-PD-1 plus pentemeric HLA-A2/NS3-1406 peptide complexes. A negative correlation between PD-1 expression and IFN-gamma production by HCV-specific T cells according to viral control is shown.

Like Retrovirus, HCV polymerase has high replication rate and lack of proofreading capacity, which permit a rapid virus escape from emerging humoral and cellular immune responses and lead to persistent infection (Chang *et al.*, 1997; Tester *et al.*, 2005). Mutation study in early HCV infection in HLA class I restricted epitopes targeted by CD8+ T cells are associated with persistence (Ray *et al.*, 2005; Timm *et al.*, 2004), which proved indirectly that HLA-restricted CD8+ T cells exert selection pressure. Furthermore, the HLA alleles can influence infection outcome (Neumann-Haefelin *et al.*, 2006).

The secretion of certain immuno-regulatory cytokines is also related with HCV persistence. IL-10 cytokine is found to increase in chronic HCV infection (Piazzolla *et al.*, 2000). In chronic HCV patients, the suppression of IFN-γ production and proliferation of virus-specific CD4+ and CD8+ T cells have been observed in livers with IL-10–producing HCV-specific CD8+ T cells (Accapezzato *et al.*, 2004). IL-10 produced by monocytes or NK cells downregulates effector T cell responses. For instance, monocytes secrete IL-10 in response to HCV core–mediated TLR2 stimulation in vitro (Dolganiuc *et al.*, 2006). IL-10 producing HCV-specific CD8+ T cells inhibits IFN-α production (Duramad *et al.*, 2003), but also promotes apoptosis of pDCs (Dolganiuc *et al.*, 2006), and induces liver infiltration of chronically infected individuals, suggesting that they modulate liver immunopathology to favor HCV persistence (Accapezzato *et al.*, 2004). In addition, intrahepatic HCV-specific IL-10 producing CD8+ T cells prevent liver damage during chronic disease (Abel *et al.*, 2006). Moreover, TGF-b is also involved in antiviral immune suppression and chronic HCV infection evolution (Alatrakchi *et al.*, 2007). To sum up these data, regulatory cytokines such as IL-10 or TGF-beta decrease liver inflammation, after affecting the protective immune response, developing a dual task. First of all, they impair T cell responses to allow viral persistence but also decrease liver damage to extend host survival.

Regulatory T cells (Tregs) are important to control the balance between host damage and viral control produced by specific immune response. In cases of excessive immune response, that could be harmful for the host, these cells can induce immune-tolerance to the viral epitopes. Tregs are derived from natural or induced T cell populations, in which natural CD4+ Tregs are generated during normal T cell development in the thymus, whereas induced Tregs are generated from mature T cells (Bluestone & Abbas, 2003). T cell subset with suppressive function, CD4+ CD25+ FoxP3+ regulatory T (Treg) cells, engages in the control of auto-immunity and immune responses, through various mechanisms including the inhibition of APC maturation and T-cell activation (Shevach, 2009). No difference has been found in the frequency of Treg cells and the extent of suppression irrespective of the outcome of the infection (Manigold *et al.*, 2006). However, higher Tregs frequency has been observed in chronic HCV infected patients than in resolved patients (Boettler *et al.*, 2005; Cabrera *et al.*, 2004; Rushbrook *et al.*, 2005; Sugimoto *et al.*, 2003). Interestingly, depletion of CD25+ cells could enhance responsiveness of the remaining HCV-specific effector cells in vitro (Boettler *et al.*, 2005; Cabrera *et al.*, 2004; Sugimoto *et al.*, 2003), which suggest a fundamental role of Tregs in the establishment of chronic HCV infection. Moreover, Treg cells are induced and proliferate in chronic HCV infection and appeared to alter liver inflammation (Zerbini *et al.*, 2008). Conversely, Programmed Death ligand-1 (PDL-1) mediated inhibition limits the expansion of Tregs by controlling STAT-5 phosphorylation (pSTAT-5) (Franceschini *et al.*, 2009), which can diminish suppressive function of Tregs, lead to viral load control and ultimately ensure long-lasting survival of the host.

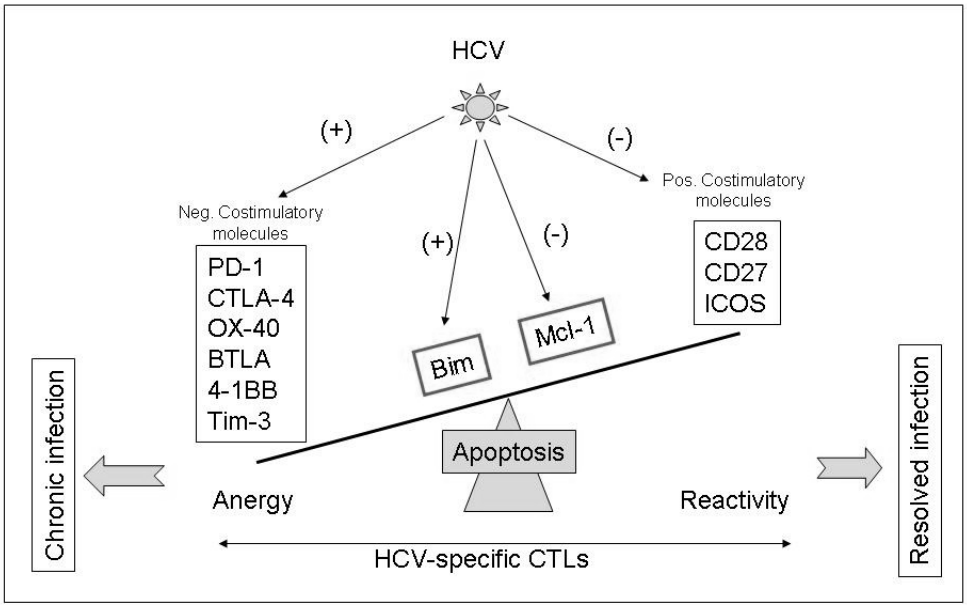

Fig. 5. Scheme showing the balance between co-stimulatory/apoptotic molecules and HCV-specific CTLs reactivity according to infection outcome. Neg.: negative. Pos.: positive. CTLs: cytotoxic T lymphocytes. HCV: hepatitis C virus. (+): possible molecules induced by HCV infection. (-): possible molecules down-regulated by HCV infection.

HCV is able to induce the up-regulation of different negative co-stimulatory molecules in order to provoke an anergic status on HCV-specific T cells. Expression of the inhibitory receptor PD-1 is one of these molecules involved in the generation of a state of exhaustion on HCV-specific CD8+ T cells during chronic HCV infection (Barber *et al.*, 2006; Larrubia *et al.*, 2009b). Importance of expression of PD-1 in HCV-specific T cell failure mechanism has been observed (Golden-Mason *et al.*, 2007; Radziewicz *et al.*, 2007), which can hinder by mutation in T cell epitopes (Rutebemberwa *et al.*, 2008). In addition, blocking of PD-1 signaling resulted in the functional restoration of blood-derived HCV-specific $CD8^+$ T cell responses in chronic infection (Penna *et al.*, 2007; Radziewicz *et al.*, 2007). However, the PD-1 alone is not sufficient in defining exhausted HCV-specific $CD8^+$ T cells during HCV infection. To restore function of HCV-specific T cells isolated from liver biopsies of infected patients, there is need of CTLA4 blockade in addition to PD-1 blockade (Nakamoto *et al.*, 2009). In addition, the co-expression of other inhibitory receptors such as 2B4, CD160, Tim-3 and KLRG1 occurred in about half of HCV-specific CD8+ T cell responses and correlate with low or intermediate level of CD127 expression, impaired proliferative capacity, an intermediate T cell differentiation stage (Bengsch *et al.*, 2010). These data indicates that HCV infection modulates different negative co-stimulatory molecules to favor the development of HCV-specific $CD8^+$ T cell exhaustion. On the other hand, HCV infection is also able to regulate pro-apototic pathways to induce HCV-specific T cell deletion in order to escape from immune response. HCV-specific CTLs from chronic patients targeting the virus express an exhausted phenotype associated to the up-regulation of the pro-apototic

molecule Bim. The activity of this molecule is contra-regulated by the anti-apoptotic molecule Mcl-1. Interestingly, the reactivity of these cells is impaired but can be restored by blocking apoptotic pathways (Larrubia *et al*., 2011).

In summary, HCV is able to impair adaptive immune response at different levels. The effector population in charged of HCV clearing is defective because HCV is able to induce on those cells anergy and apoptosis (Fig.-5). Moreover, HCV is able to escape humoral response and cellular response by escape mutations in immunodominant epitopes. Finally, HCV is also quite efficient in the impairment of the specific T helper response, which is essential to organize the humoral and cellular response. To perform all these immune-escape strategies, HCV takes advantage of the pro-anergenic environment of the infected liver, because HCV-specific T cell priming at this level is not efficient to develop adequate effector cells. As it was commented for HBV infection, HCV immune response restoration could be an interesting therapeutic tool to help in viral clearance in chronic patients.

4. Accepted model of HBV&HCV pathogenesis

As previously commented, specific CTLs play a central role in HBV&HCV immunopathogenesis. These cells are able to kill some infected hepatocytes inducing a minor liver damage, but also they secrete type-I cytokines responsible for non-cytopathic virus clearing. To attract these cells into the liver the infected hepatocytes secrete another kind of cytokines called chemokines. The migration of lymphocytes to the liver is a complex process including adhesion, rolling, triggering, and transendothelial migration. Chemokines and their receptors play an essential role in this multistep pathway (Springer, 1994). During primoinfection, when the adaptive immune system is not able to control infection, the infected hepatocytes continue secreting chemokines to try to attract more defensive cells. In viral chronic hepatitis, the expression of different chemokines in the liver has been described. CXCL-10 is increased in the liver and peripheral blood during viral chronic hepatitis (Larrubia *et al*., 2007; Shields *et al*., 1999; Tan *et al*., 2010; Wang *et al*., 2008). This molecule is produced by hepatocytes and sinusoidal endothelial cells. Moreover, CXCL9 and CXCL11 are also increased in serum and liver of subjects with chronic viral hepatitis (Bieche *et al*., 2005). CXCL9 is detected primarily on sinusoidal endothelial cells, while CXCL-11 is produced mainly by hepatocytes (Apolinario *et al*., 2002). CCL5 intrahepatic expression is also elevated in viral chronic hepatitis and is produced by hepatocytes, sinusoidal endothelial cells and biliary epithelium. Finally, several studies have reported an increased level of CCL3 and CCL4 either in the liver or in serum. These molecules are detected on endothelial cells, on some hepatocytes and biliary epithelial cells (Apolinario *et al*., 2004). The expression of all these chemokines in the liver can be induced directly by viral proteins. Previous reports have shown a high hepatocyte synthesis of CXCL10, CXCL9 and CCL5, induced by some HCV proteins such as NS5A and core (Apolinario *et al*., 2005), although a recent in-vitro study suggests that HCV proteins could also decrease CCL5 and CXCL10 genes expression (Sillanpaa *et al*., 2008). All these chemokines recruit T cells with a Th1/Tc1 phenotype, expressing specific chemokine receptors such as CCR5 and CXCR3. The non-ELR-CXC chemokine attracts CXCR3 expressing T cells while CC chemokine attract CCR5 expressing T cells to the liver. Consequently, in viral chronic hepatitis, an intrahepatic enrichment of CCR5 and CXCR3 expressing T cells, located in hepatic lobule and portal tracts has been shown, while these populations are very infrequent in uninfected subjects (Bertoletti & Maini, 2000; Larrubia *et al*., 2008) (Fig.- 6).

Persistent HBV&HCV infection is characterized by a non-specific inflammatory infiltrate in the liver, mainly of CD8+ cells (Sprengers *et al.*, 2005), responsible for liver damage. These cells are attracted by the interaction between the intrahepatic secreted chemokines and the chemokine receptors expressed on T cells. Actually, previous reports have shown a correlation between liver inflammation and liver infiltrating CXCR3/CCR5 expressing T cells. The frequency of these cells was positively correlated with portal and lobular inflammation but not with liver fibrosis (Larrubia *et al.*, 2007). These data suggest that CCR5 and CXCR3 could play an important role in chronic liver damage by means of inflammatory T cells recruitment into the liver. Moreover, several previous studies have also shown a correlation between liver inflammation and chemokine levels. Intrahepatic CXCL10 mRNA levels are associated with intralobular inflammation (Harvey *et al.*, 2003). Similarly, CXCL9 and CXCL11 correlate with the grade of liver inflammation (Helbig *et al.*, 2004). Furthermore, CC chemokines are also correlated with the intrahepatic inflammatory activity (Kusano *et al.*, 2000). Clearly, intrahepatic CCL5 positive cells correlate with the inflammatory activity. Bearing in mind all the previous data it is possible to speculate that

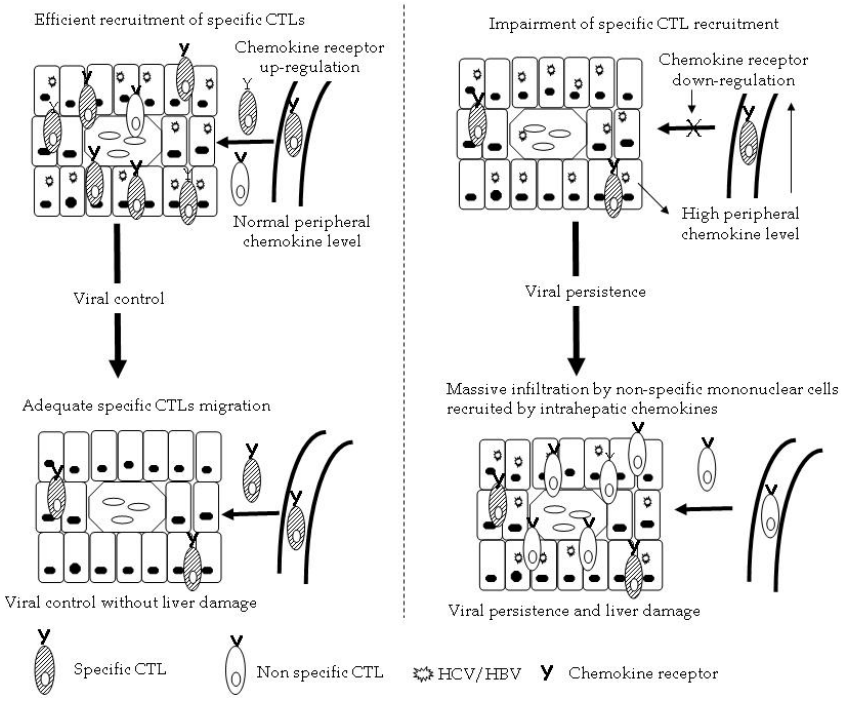

Fig. 6. Scheme showing the role of T cell intrahepatic recruitment according to the degree of liver damage and viral control. In resolved HBV/HCV infection an adequate effector T cell response is attracted to the liver to clear the virus. After that, a memory T cell population is continuously patrolling the liver to keep under control viral traces. Nevertheless, in persistent infection after specific T cells failure to control infection, a non-specific immflamatory infiltrate is sequestered into the liver, responsible of the persistent liver damage.

chemokines are secreted in the infected liver to attract an adaptive immune response able to clear the virus. Unfortunately, when the specific response fails these chemokines also attract non-specific mononuclear and polymorphonuclear cells, which are not able to remove the virus but produce liver inflammation (Kakimi *et al.*, 2001). Therefore, as chemokines are nonspecific chemoattractants, intrahepatic inflammatory infiltrate during chronic infection is mainly non-virus-specific and consequently unable to eliminate the infection, but able to produce cytokines capable of initiating and perpetuating hepatic fibrogenesis (Bertoletti & Maini, 2000; Bertoletti *et al.*, 2010; Friedman, 2003; Larrubia *et al.*, 2008).

5. Mechanisms to restore adaptive immune response in HBV&HCV infection

Bearing in mind that specific T responses are essential to control HCV during natural immune response, several studies have been performed to analyze the role of different therapeutic approaches on T cell response to know whether it is possible to reverse T cell dysfunction in-vivo. Longitudinal analysis of HBV-specific responses during IFN-α treatment did not show a significant increase of these responses during treatment (Sprengers *et al.*, 2007). Nevertheless, it was observed an improvement after treatment in patients with resolved infection (Carotenuto *et al.*, 2009). During HCV infection, the same scenario was observed (Barnes *et al.*, 2002), although some studies have demonstrated a restoration of T cell response in sustained viral responders (Kamal *et al.*, 2002). However, patients presenting a better HCV-specific CD8 cell proliferative potential at baseline, are more likely to present a rapid and sustained viral response. Moreover, after treatment a HCV-specific T-cell response enhancement is observed in sustained viral responders (Pilli *et al.*, 2007). The absence of T cell reactivity improvement during treatment could be due to the direct anti-proliferative effect of IFN-α. Obviously, this effect could counteract the positive consequence of decreasing viral pressure on specific-T cells during treatment. Nevertheless, these data also could suggest that is important to restore a specific T cell response, at least at the end of treatment, to keep under control residual viral traces. In HBV infection, several papers dealing with the role of nucleot(s)ide analogues (NUCs) treatment on anti-HBV immune response have shown that they are able to reconstitute temporally HBV-specific CD4 and CD8 responses (Boni *et al.*, 1998; Cooksley *et al.*, 2008). Moreover, these treatments can decrease the frequency of Tregs during treatment (Stoop *et al.*, 2007) and specifically to decrease the ratio Treg:Th17 (Zhang *et al.*, 2010). These data suggest that the NUCs are controlling the infection not only through an anti-viral effect but also helping to restore specific immune response. In any case, all these effects on specific T cells are partial and limited in time. For that reason other immunoregulatory therapeutic approaches are being considered. Several pre-clinical studies have been performed to try to restore HBV/HCV specific responses in-vitro and in animal models. Modulation of negative co-stimulatory molecules, in addition to blocking immunosupressive cytokines could be promising strategies to restore an effective T cell response. The modulation of negative co-stimulatory molecules, such as PD-1, CTLA-4, Tim-3, has shown in-vitro to increase specific-T cell reactivity. This can be also enhanced using Abs to block the regulatory cytokine IL-10. Experts in immunotherapy have suggested that after restoring a T cell response could be necessary to boost that response using a therapeutic vaccine. Although these results seem to be quite promising, the blockade of negative co-stimulatory pathways in addition to IL-10 could lead to the development of autoimmune diseases, which could prevent the use of this strategy as a therapeutic tool in humans. Therefore, more research is necessary in this field

before these strategies are suitable for the treatment of chronic viral infections (Ferrari, 2008; Fisicaro *et al.*, 2010; Larrubia *et al.*, 2011; Nakamoto *et al.*, 2009).

6. Conclusions

HBV & HCV are two hepatotropic non-cytopathic viruses able to develop a chronic liver disease. The innate immune response is defective in both infections, residing the viral control in the efficacy of adaptive immune response. HBV&HCV specific CTL response play a central role in viral control through cytopathic and non-cytopathic mechanisms. Nevertheless, during persistent infection, adaptive response is impaired due to exhaustion and deletion. Several in-vitro strategies have shown to be effective in its restoration but it is necessary more research before these approaches can be applied to clinical practice. Finally, when the virus is not controlled by adaptive response a non-specific inflammatory infiltrate is attracted to the liver which is responsible for the persistent low-grade liver damage, allowing the generation of liver fibrosis during disease progression.

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