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HETEROGENEITY OF THE EXHAUSTED HBV-SPECIFIC CD8 T CELL POPULATION AS DEFINED BY FUNCTIONAL/PHENOTYPIC PROFILING

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RIASSUNTO

Il virus dell'epatite B (HBV) rappresenta uno dei principali problemi di salute pubblica a livello globale. L'attuale terapia antivirale per l'infezione cronica da HBV si basa sul trattamento con interferone alfa peghilato (PEG-IFN-α) a durata definita o sulla somministrazione a lungo termine di analoghi nucleos(t)idici (NUC). A causa della bassa percentuale di clearance di HBsAg e di sieroconversione anti-HBs ottenibile con i farmaci attualmente disponibili, lo sviluppo di strategie terapeutiche innovative che possano accelerare la perdita di HBsAg e la produzione di anticorpi anti-HBs rappresenta un'esigenza clinica prioritaria.

I pazienti con infezione cronica da HBV presentano difetti funzionali delle risposte immunitarie antivirali, che tendono ad approfondirsi con il passare del tempo, contribuendo in tal modo alla persistenza virale. In questa prospettiva, la modulazione delle risposte T linfocitarie HBV-specifiche mediante l'utilizzo di composti ad azione immunomodulante rappresenta una strategia potenzialmente efficace nel tentativo di implementare la risposta immunitaria nei pazienti con infezione cronica da HBV.

Sulla base di studi precedenti eseguiti nel modello murino di infezione da LCMV e nell'infezioni croniche da HCV e HIV nell'uomo, è noto come i linfociti T virus-specifici rappresentino una popolazione cellulare eterogenea caratterizzata da distinti subsets T linfocitari coesistenti con vario grado di exhaustion e con differente risposta al blocco di pathway inibitori. Per approfondire il fenomeno dell'esaurimento T linfocitario nei pazienti con infezione cronica da virus B, risulta di fondamentale importanza la comprensione di quanto eterogenee siano le popolazioni cellulari HBV specifiche e se l'analisi dei differenti subsets T linfocitari possa essere utile per predire la risposta a

strategie terapeutiche correttive immunomodulanti.A questo proposito, l'analisi simultanea di fattori di trascrizione, recettori coinibitori e molecole di differenziazione, come ad esempio PD1, CD39, TOX, CD127, Bcl-2 e TCF1, ha permesso di distinguere subsets di linfociti T con migliore attività antivirale in differenti categorie di pazienti.L'analisi della co-espressione dei suddetti marcatori ha consentito di definire specifici *subsets* di linfociti con vario grado di disfunzione, che potrebbero essere variamente suscettibili di strategie correttive della loro funzione. Più precisamente, focalizzando l'attenzione sull'espressione di PD1 e CD127, i dati mostrano come i linfociti CD8 HBV-specifici destramero-positivi si distribuiscano in modo differente nelle diverse casistiche cliniche di pazienti: la sottopopolazione cellulare $PD1^{high}$ $CD127^{low/-}$ sembra essere prevalentemente espressa dai soggetti con infezione cronica non trattati, configurando un profilo fenotipico più "*exhausted*"; al contrario il subset cellulare $PD1^{low/-}$ $CD127^{high}$ sembra caratterizzare i linfociti CD8 HBV-specifici di pazienti che hanno sviluppato anticorpi anti-HBs come pure quelli CD8 influenza-specifici. Infine la sottopopolazione $PD1^{high}$ $CD127^{high}$ risulta presente in tutti i gruppi di pazienti, indipendentemente dalle condizioni cliniche e dal livello di controllo anti-virale.

Lo studio funzionale ha evidenziato un livello percentuale di produzione di citochine antivirali *ex vivo* globalmente più basso nei pazienti con epatite cronica rispetto ai pazienti che hanno conseguito controllo anti-virale. In particolare, la produzione di citochine anti-virali è risultata essere significativamente meno efficiente all'interno del subset T linfocitario $PD1^{high}$ $CD127^{low/-}$ rispetto a quella rilevata nei subsets $PD1^{low/-}$ $CD127^{high}$ e $PD1^{high}CD127^{high}$. Al contrario, la produzione citochinica è risultata essere omogeneamente espressa all'interno dello stesso subset $PD1^{low/-}$ $CD127^{high}$ per tutte le categorie di pazienti.

L'analisi comparativa dei risultati fenotipici e funzionali ha evidenziato come alcuni pazienti con infezione cronica, caratterizzati da un profilo fenotipico meno "*exhausted*", mostrassero un profilo paragonabile ai soggetti che sono andati incontro a guarigione.

Infine, per implementare questo algoritmo predittivo sono stati analizzati *ex vivo* ulteriori marcatori fenotipici di differenziazione e co-inibitori quali CD39, TOX, Bcl-2 e TCF1.

In linea con studi precedenti, l'espressione del marcatore di esaurimento CD39 è risultata rilevabile prevalentemente nei pazienti infettati cronicamente, evidenziando una correlazione statisticamente significativa tra i livelli di questo marcatore e la frequenza del subset più "*exhausted*" $PD1^{high}$ $CD127^{low/-}$.

Analogamente, anche il marcatore di esaurimento TOX è risultato maggiormente espresso nella sottopopolazione più disfunzionale $PD1^{high}$ $CD127^{low/-}$ rispetto a quella più protettiva $PD1^{low/-}$ $CD127^{high}$. Al contrario, il fattore di trascrizione TCF1, coinvolto nella differenziazione cellulare e nel mantenimento di memoria proteggente, così come la molecola anti-apoptotica Bcl-2 sono risultati significativamente up-regolati nei subsets $PD1^{low/-}$ $CD127^{high}$ e $PD1^{high}CD127^{high}$ rispetto alla sottopopolazione più esaurita $PD1^{high}$ $CD127^{low/-}$.

La frequenza relativa di differenti subsets linfocitari con distinti gradi di exhaustion in singoli pazienti potrebbe permettere di identificare una coorte di pazienti con infezione cronica caratterizzati da un sistema immunitario più efficiente, i quali potrebbero mostrare una migliore possibilità di risposta a strategie terapeutiche immunomodulanti. Tali informazioni potrebbero essere applicate per lo sviluppo di strategie di ripristino immuno-terapeutico per la cura dell'infezione cronica da HBV e per monitorare in modo ottimale i risultati ottenuti con i nuovi composti antivirali.

ABSTRACT

Chronic hepatitis B virus (HBV) infection represents a public health problem worldwide with approximately 250 million people chronically infected at increased risk of developing liver cirrhosis and cancer.

Currently available antiviral treatments for chronic HBV infection are based on nucleos(t)ide analogues (NUC), which efficiently suppress viral replication but frequently require a lifelong administration and have limited effect on HBsAg concentrations, and on interferon alpha (IFN), which induces a sustained off-treatment viral suppression in only a minority of patients and is associated with severe side effects. Therefore, the development of new antiviral strategies to provide durable HBsAg loss with limited treatment duration is highly needed.

During chronic HBV infection, virus-specific T lymphocytes are poorly responsive to antigenic stimulation and their responses are deeply dysfunctional. This functional impairment is the consequence of different inhibitory mechanisms, including the persistent exposure to high antigen loads, and the resulting exhaustion state is widely believed to contribute to viral persistence.

Therefore, functional T cell reconstitution strategies represent possible candidates for the development of novel cure strategies with finite duration of treatment to improve the management of chronic patients and the prevention of HBV-associated hepatic complications.

Available data from mouse and other human models of chronic virus infection indicate that exhausted virus-specific CD8 T cells are a heterogeneous dysfunctional population composed of variably differentiated subsets with different response to checkpoint

blockade. In order to further characterize this phenomenon in HBV infection, we assessed how heterogeneous are exhausted HBV-specific T cells in chronic HBV patients and whether the analysis of exhausted CD8 T cell subsets co-existing in individual chronic patients can be helpful to predict response to immune modulators.

For this purpose, a molecular characterization of T cell exhaustion in the HBV-specific T cell population in CHB patients was conducted by assessing the co-expression of transcription factors, co-inhibitory receptors and differentiation molecules (e.g. PD1, CD39, TOX, CD127, Bcl-2 and TCF1) that allowed to distinguish T cell subsets with different antiviral activity in different cohorts of CHB patients. In particular, co-expression levels of the inhibitory receptor PD-1 and the T cell memory-related marker CD127 distinguished three different T cell subsets: a $PD1^{high}$ $CD127^{low/-}$ cell subset selectively prevalent in naïve viremic patients; a $PD1^{high}$ and $CD127^{high}$ subset present in both CHB and in spontaneous resolver patient cohorts, irrespective of the clinical condition and the level of virus control; a $PD1^{low/-}$ $CD127^{high}$ subset associated with a more protective role in consideration of the preferential detection in patients who spontaneously controlled infection.

To confirm that the prevalence of the $PD1^{low/-}$ and $CD127^{high}$ over $CD127^{high}$ $CD127^{low/-}$ subset in individual patients is an indication of better protection, T cell cytokine production was assessed in the different groups of patients. Total *ex vivo* cytokine levels (IFN-γ and TNF-α) were significantly lower in chronic patients as compared to resolvers and also to flu-specific CD8 cells. The $PD1^{high}CD127^{low/-}$ T cell subset was less efficient than the $PD1^{low/-}CD127^{high}$ subset in terms of total cytokine production. Moreover, cytokine production by $PD1^{low/-}CD127^{high}$ cells was similar in chronic patients and in NUC resolvers and subjects with spontaneous seroconversion.

This phenotypic and functional T cell characterization allowed the identification of a distinctive CHB patient cohort with features more similar to resolved patients.

To improve the predictive capacity of these profiles, other markers were studied. The PD-$1^{high}CD127^{low/-}$ cells showed a significant correlation with the levels of the ecto-nuclease CD39, which leads to the generation of immune suppressive adenosine. On the other hand, the anti-apoptotic marker Bcl-2 and the memory-related transcription factor TCF1 were highly expressed within the $CD127^{high}$ subsets in both chronic and spontaneously resolved patients.

Accordingly, the HMG-box exhaustion-associated transcription factor TOX displayed significantly higher expression in the more impaired $PD1^{high}$ $CD127^{low/-}$ cell subset in comparison with the less dysfunctional $PD1^{low/-}$ $CD127^{high}$ T cell subpopulation in chronically infected patients.

The relative predominance of more or less exhausted T cell subsets seems to dictate the level of functional efficiency of the overall anti-viral CD8 T cell population and those patients who harbor a less inhibited virus-specific immune system with predominance of less exhausted CD8 T cell subsets may represent a CHB patient cohort with a better chance of response to immune modulatory interventions. This novel pathogenetic information could be applied for the development of future immune reconstitution strategies to cure HBV infection and to better monitor the results obtained with new anti-viral compounds.

INTRODUCTION

1. Hepatitis B virus

1.1 Genome, replication and genotypes.

The hepatitis B virus (HBV) has a small partially double stranded circular DNA genome and it is a member of the Hepadnaviridae family, which includes hepatotropic viruses. The HBV virion consists of a nucleocapsid core (HBcAg), enclosed within a lipoprotein coat (also called envelope) containing the surface antigen (HBsAg). By electron microscope, the HBV structure appears like a spherical, double shelled particles, 42–47 nm as diameter, and it is called *Dane particles*.

HBV genome encodes four partially overlapping open reading frames (ORFs) resulting in structural and non-structural viral proteins. DNA Polymerase, the central enzyme in genome replication, is encoded by the P (Pol) ORF and it has reverse transcriptase activity similar to retroviruses (Figure 1). Indeed, Hepatitis B is one of the few known non-retroviral viruses which use reverse transcription as a part of its replication process. The C (core) and pre-C regions encode the structural protein of the nucleocapsids (HBcAg), and the non-structural e antigen (HBeAg), which is released into the serum of the infected patient. The S (surface) region encodes the large, medium, and small envelope glycoproteins containing PreS1, PreS2 and HBs antigenic reactivities. The X region encodes the multifunctional X protein (HBx), expressing transcription regulatory properties. Others regulatory elements contain promoters, enhancers and signals for polyadenylation and encapsidation[1–4].

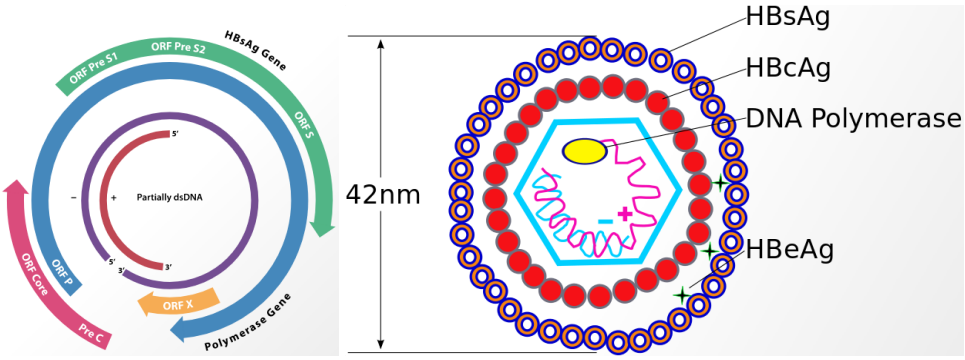

Figure 1. HBV viral particles and genome structure.
On the left, the structure of the HBV genome: in the inner part DNA strands are shown, externally the different regions of the HBV genome coding for the structural and non-structural proteins of the virus are represented by colored arrows. On the right, a schematic overview of a viral particle in which have been highlighted protein constituents: envelope (HBsAg) and nucleocapsid (HBcAg and HBeAg) antigens and DNA polymerase associated with the viral genome.

During replication, the HBV genome can integrate into the chromosomal DNA of hepatocytes providing a distinct ability to the virus to persist in infected cells and expand clonally. Indeed, the nucleocapsid enters into the nucleus of the infected hepatocyte, where the HBV DNA is transformed in a covalently closed circular DNA (cccDNA), a mini-chromosome which works as a template for the viral protein synthesis

Thus, serum indicators of viral replication are HBV DNA, the S1 proteins (HBsAg) and the soluble hepatitis B e antigen (HBeAg), released from hepatocyes[3].

HBV genomic DNA sequencing revealed the presence of several viral genotypes with a distribution that is dependent on the geographical areas (Figure 2). Moreoever, HBV genotypes are correlated with disease and clinical progression, response to therapy, and prognosis. So far, 10 HBV genotypes (A-J) have been determined with > 8% differences in nucleotides, and further divided in sub-genotypes with 4%-8% nucleotide differences.

Genotype A is endemic in sub-Saharan Africa, Northern Europe, and Western Africa; the B and C are diffuse in Asia; the C is common in Southeast Asia; genotype D is widespread in Africa, Europe, Mediterranean countries, and India; the G is common in France, Germany, and USA; and genotype H is reported in Central and South America. In Vietnam and Laos is common genotype I. The new genotype J has been reported in in Ryukyu Islands (Japan)[5].

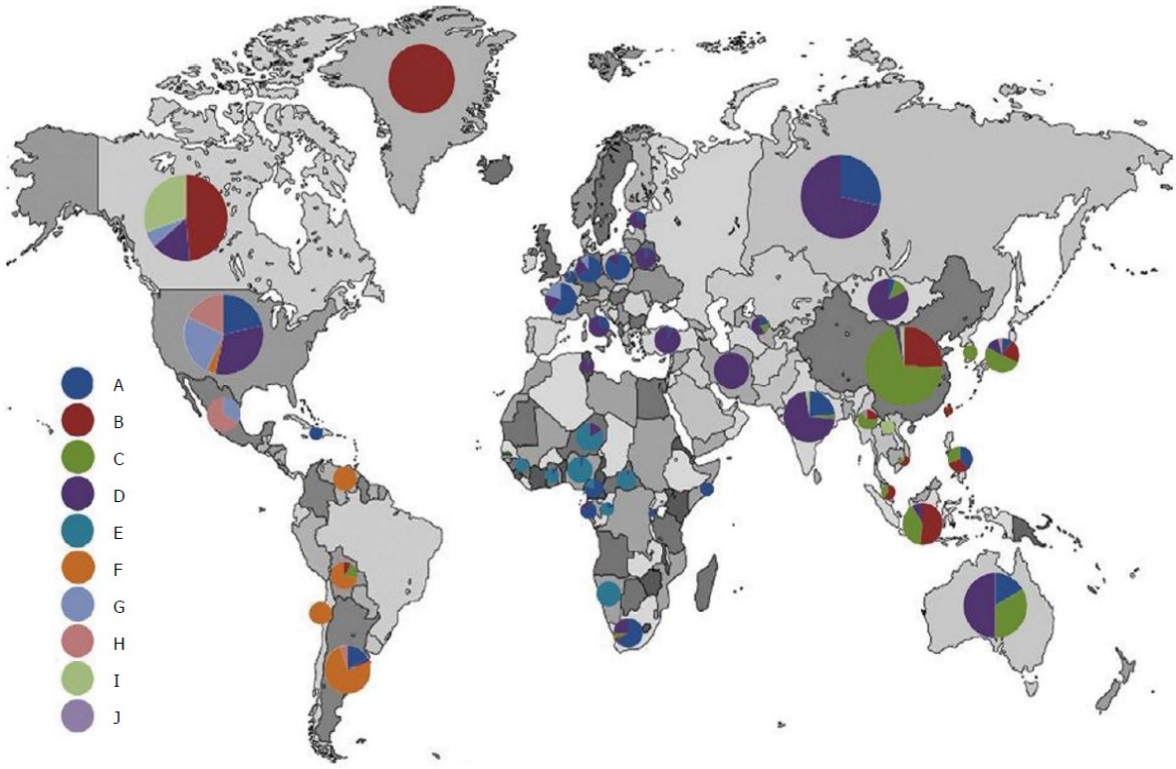

Figure 2. Worldwide HBV genotype distribution.
Source: Shi W, Zhang Z, Ling C, Zheng W, Zhu C, Carr MJ, Higgins DG. Hepatitis B virus subgenotyping: history, effects of recombination, misclassifications, and corrections. Infect Genet Evol 2013

1.2 Epidemiology and public health burden

The hepatitis B virus is the most common hepatic virus responsible for chronic infections affecting humans, therefore representing a global public health problem.

Approximately 30% of people displays serological signs of present or past HBV infection[6,7]. According to the World Health Organization, more than 2 billion individuals have contracted HBV infection and more than 350 million of them are infected with chronic hepatitis B. Chronic hepatitis caused by HBV infection is a major causes of cirrhosis and hepatocellular carcinoma, with an estimated 780,000 deaths each year caused by complications due to liver damage consequent to the infection.

The incidence of HBV infection and the mode of transmission vary widely across the world, depending on the different geographical areas; thus, high, intermediate and low endemic regions can be distinguished (Figure 3)[8]. High endemic regions, where the prevalence of infection is equal to or greater than 8%, correspond mainly to Sub-Saharan Africa and South-East Asia; intermediate endemic regions, with a prevalence of HBsAg between 2-7%, are mainly India and Middle Eastern countries; low endemic regions with a prevalence of infection of less than 2% include Western Europe, North America and Australia[8,9]. Italy ranks among the regions with low endemic levels, with a prevalence of chronic infection around 1%, which in recent decades undergoes a clear infections decrease, mainly achieved through the introduction of mandatory vaccination for newborns since 1991[10].

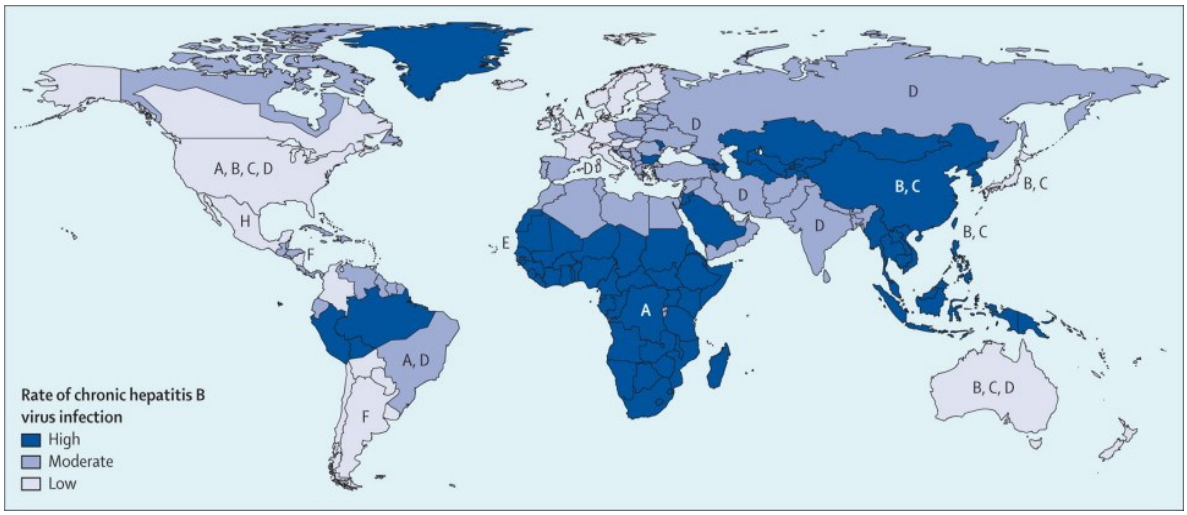

Figure 3. Prevalence map of HBV infection in the world . The different colors highlight the geographical areas of high, intermediate and low HBV endemic levels. Source: Christian Trépo, Henry L Y Chan, Anna Lok - Hepatitis B virus infection - Lancet 2014.

1.3 Natural History of Infection

The natural cycle of chronic HBV infection is a dynamic process that is divided into different phases derived from the interaction between the virus, hepatocytes and the host's immune response. Five phases are generally considered and distinguished on the basis of specific biochemical, serological and virological features - e.g. HBeAg, HBV DNA levels, alanine aminotransferase (ALT) values and the presence or absence of liver inflammation. To note these phases do not occur in all patients with chronic HBV hepatitis, nor do they necessarily occur sequentially[11].

In particular, during acute HBV infection the symptoms and the outcome depend on age of infection. In fact, infants and children do not show symptoms, conversely around 70% of adults have subclinical hepatitis and 30% have an icteric hepatitis. Those HBV acute infections that progress toward a fulminant hepatitis are around 1% and the mortality is roughly 80% in the absence of liver transplantation.

In people who spontaneously recover, HBsAg appears in the serum 2–10 weeks after HBV exposure, before ALT increase and before the onset of symptoms, disappearing in 4–6 months with HBsAg seroclearance and Anti-HBs production (Figure 4A). Indeed, HBsAg persistence beyond 6 months defines the progression to chronicity (Figure 4B).

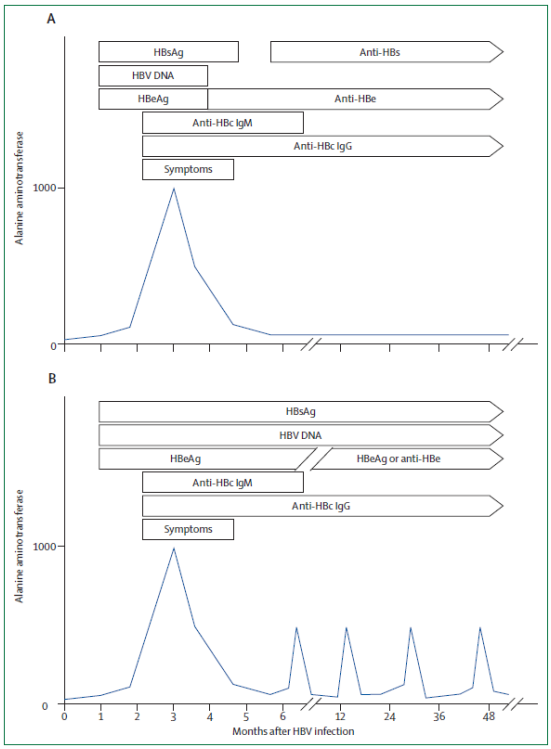

Figure 4. HBV markers during natural course resolved acute HBV infection (A) and transition toward chronic HBV infection (B). Source: Christian Trépo, Henry L Y Chan, Anna Lok - Hepatitis B virus infection - Lancet 2014.

Thus, when the HBV exposure occurs during infancy or childhood, the initial chronic phase (Table 1: PHASE 1) is featured by HBeAg positivity, high levels of HBV DNA (>10^7 IU/mL) and of HBsAg (>10^5 IU/mL) with normal ALT concentrations, associated to a minimum liver inflammation. This chronic infection can continue for 20–40 years with no disease development, and the rate of HBeAg seroconversion is quite low. The lack of liver damage even though with high viraemia, due to immune tolerance, is probably due to clonal elimination of HBV-specific T cells in the fetus through in-utero HBeAg exposure [12].

The second phase starts with the loss of immune tolerance (Table 1: PHASE 2) and corresponds to HBeAg+ chronic infection. Moreover, a decrease in HBV DNA and an increase in ALT concentrations are observed together with lower concentrations of HBsAg (10^3–10^4 IU/mL)[13]. Furthermore, around 10–20% of patients develops anti-HBe (HBeAg seroconversion) within a year[14]. Moreover, ALT increment with failed clearance increases the possibility of liver cirrhosis and liver carcinoma (HCC)[15].

Thus, effective HBeAg seroconversion with HBV DNA elimination and normalization of ALT levels leads to an inactive phase (Table 1: PHASE 3).This inactive phase 3 is characterized by low/ undetectable HBV DNA concentration (<2000 IU/mL) and the HBsAg levels decrease to 10^2–10^3 IU/mL. The spontaneous HBsAg seroclearance is around 0.5–1% per year[16], those patients with HBsAg level < 1000IU/mL undergo more likely HBsAg seroclearance[17] with diminished possibility to develop cirrhosis and HCC with respect to those having higher HBsAg levels[18].

Approximately 20–30% of patients undergo HBV infection reactivation, with elevated viral DNA or ALT levels and with absence of HBeAg – chronic HBeAg-negative infection phase (Table 1: PHASE 4)[19]. HBeAg-negative subjects with active disease, show a higher risk to develop cirrhosis and HCC[15].

The next stage of HBV infection, HBsAg-negative phase, is defined by not detectable HBsAg and the presence in the serum of antibodies specific for HBcAg (anti-HBc); conversely, antibodies for HBsAg (anti-HBs) have not been necessary developed. This phase is also called “occult HBV infection” (Table 1: PHASE 5), and patients exhibit normal ALT levels and usually, the absence of HBV DNA in the serum. In addition, in the liver HBV DNA (cccDNA) can be observed frequently[20].

Moreover, the HBsAg disappearance before occurring cirrhosis is correlated with a reduced cirrhosis risk, decompensation and hepatocellular carcinoma, and a survival enhancement. Unfortunately, if cirrhosis occurs before HBsAg loss, patients retain the risk to develop HCC. Furthermore, immunosuppressive therapies may drive HBV reactivation in these subjects[20].

PHASE	1	2	3	4	5
New Terminology	HBeAg positive Chronic infection	HBeAg positive Chronic hepatitis	HBeAg negative Chronic infection	HBeAg negative Chronic hepatitis	Resolved HBV infection
Old Terminology	Immune tolerant	HBeAg positive CHB	Inactive carrier	HBeAg negative CHB	HBsAg negative / anti-Hbcore positive
HBsAg	High	High / Intermediate	Low	Intermediate	Negative
HBeAg	Positive	Positive	Negative	Negative	Negative
HBV DNA	> 10^7 IU/ml	10^4 - 10^7 IU/ml	< 2000 IU/ml	> 2000 IU/ml	< 10 IU/ml
ALT	Normal	Elevated	Normal	Elevated	Normal
Liver disease	None/ minimal	Moderate/ severe	None	Moderate/ severe	None
Disease progression	Low	Moderate to high	No, very low	Moderate to high	None
Treatment	Not indicated	Indicated	Not indicated	Indicated	Not indicated but prophilaxis for selected cases

Table 1. New classification of HBV infection phases, according to the recent "EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection".
Source: Table kindly provided by Prof. M. Levrero (oral presentation, 2018)

2. Therapies and Therapeutic Strategies

2.1 Current antiviral therapies: pegylated interferon-alpha and nucleos(t)ide analogues

The eradication of the infectious agent is the ideal target for the treatment of any chronic infection. Unfortunately, with respect to HBV, the complete eradication is hard to achieve with the currently available therapy options for CHB, because covalently closed circular DNA (cccDNA) hides in host hepatocytes[21] with the possibility of relapse during immunodepression conditions. Therefore, to date the main goal of the current therapy guidelines for patients with chronic HBV infection, is to improve survival and quality of life by preventing fibrosis, and consequently HCC development[11].

Current antiviral therapy for chronic HBV infection is based at present either on short-term pegylated interferon-alpha (PegIFNa) treatment or on long-term administration of nucleos(t)ide analogues (NUC)[22] (Figure 5).

IFNs are naturally occurring cytokines in different forms, such as IFN-α produced by lymphocytes[23], and were identified for the first time in 1957. IFNs are able to interfere with viral activity and are used for their immunomodulatory, anti-proliferative, and antiviral efficacy.

IFN-α exerts its direct immunomodulatory functions through the binding to its cellular receptor, by activating secondary messengers and increasing the production of proteins crucial for the cellular defense against viruses, such as HBV. Enhancement of antigen presentation to the immune system, activation of natural killer (NK) cells and other immune cells, and increased production of cytokines represent the main mechanisms by which the IFN exerts its immunomodulatory effects; while the antiviral actions arise through mRNA viral degradation, inhibition of viral protein synthesis and protection from infection of cells[24].

In patients with chronic HBV infection IFN-α can induce viral suppression in a small percentage of treated subjects and its administration is frequently followed by side effects. The aim of this therapy is to transform an active into an inactive infection but this therapeutic strategy does not necessarily leads to a complete inhibition of HBV replication[25,26].

NUCs (*lamivudine, telbivudine, adefovir, entecavir,* and *tenofovir*) are very effective in decreasing production of virions through inhibiting the viral polymerase activity and reducing viral nucleocapsids recycling to the nucleus of infected cells, but the major

drawback of this treatment is however its duration, in fact it can be safely interrupted only after anti-HBs seroconversion to avoid HBV reactivation[11].

Since anti-HBs production is a slowed event in the HBV chronic infection, most patients undergo to a life-long therapy with high costs for the national health system and with possible side effects during all their life.

Thus, an urgent medical need is to reveal new reliable predictors of infection immune control in NUC-treated patients, with the aim to accelerate an earlier and safe NUC withdrawal based on anti-HBs appearance without the risk of hepatic flares, which were observed after NUC suspension without HBsAg clearance[27–32].

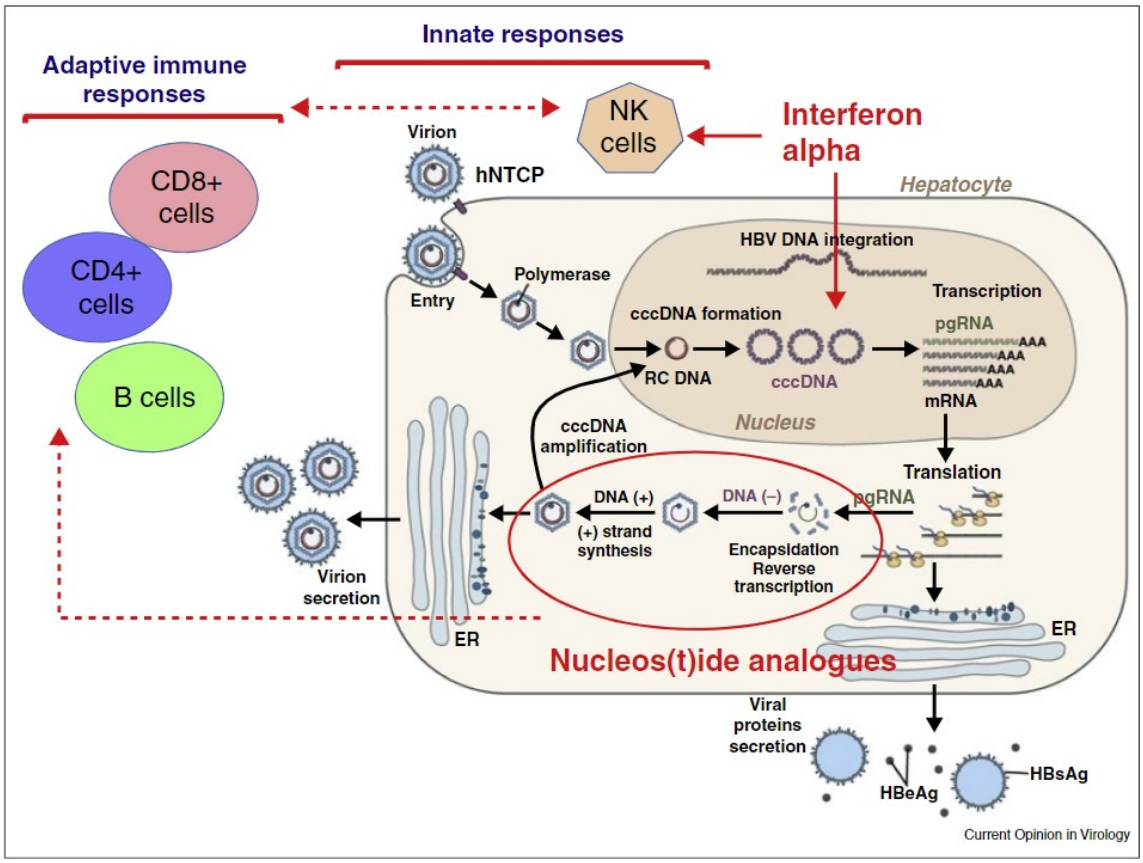

Figure 5. Scheme for the action mechanism of antivirals for HBV chronic infection. Interferon-α induces NK cell activity and down-regulates HBV specific cell activity. In experimental models, its antiviral effect has been attributed to the repression of viral cccDNA transcription and to the partial elimination of the cccDNA pool. Nucleos(t)ide analogs (NUCs) block the reverse transcriptase subunit of the DNA polymerase, thus reducing the circulating viral DNA. Prolonged viral suppression obtained with NUCs induces HBV-specific T cell restoration. These antivirals do not eradicate viral cccDNA from infected hepatocytes.

Source: Zoulim F, Lebossé F, Levrero M. Current treatments for chronic hepatitis B virus infections. Current Opinion in Virology. 2016

2.2 Immunotherapy and New Therapeutic Strategies

The primary objectives for the development of new therapies are based on the reduction of timing administration, on the low risk of pharmacological resistance development and on the prevention of side effects, with the aim to obtain a total clearance of HBsAg and, when possible, a complete viral eradication.

Recent studies are trying to identify compounds capable of intervening on various aspects of the HBV life cycle and, in this regard, the potential therapeutic targets could include: inhibition of viral entry in the hepatocytes, destruction of the viral nucleocapsid, the direct intervention on the cccDNA and the prevention of the HBsAg release. Other currently ongoing therapeutic strategies concern the intervention on the host's immune system, with the aim of stimulating the immune response against the virus and making antiviral treatment more effective[33]. With the aim to stimulate the innate immune response, toll-like receptor analogues have been studied. Toll-like receptors (TLRs) play a key role in the recognition of external pathogens, both bacteria and viruses, and are considered as part of the innate immune response. TLR3, 7/8 and TLR9 are a TLR subfamily that recognize endosomal viral nucleic acids (dsRNA, ssRNA and CpG motifs respectively) and induce type I IFN production[34].

Besides, with the aim of stimulating adaptive immune responses at the level of CD4+ and CD8+ T cells, possible therapeutic vaccines are being tested. The purpose of therapeutic vaccines is to restore or induce T cell responses by improving the quality of antigen presentation by specialized cells, in a context in which there is a substantial production of antigens, which is responsible for the peripheral and intrahepatic T cell exhaustion, and an inadequate antigen presentation by hepatocytes (in which instead HBV replication

occurs)[33,35]. In this perspective, the properly orchestrated activation of antiviral immunity could be fundamental to obtain the so-called “functional cure” for HBV infection. In fact, patients who controlled HBV displayed a coordinated activation of both humoral and cellular immune HBV-specific responses. The strength of HBV-specific immune responses is confirmed by those chronic patients who underwent to bone marrow transplantation from resolved HBV donors (with HBV-specific memory B and T lymphocytes), became negative for HBsAg[36]. Hence, restoring the immune-mediated mechanisms can lead to a natural disease recovery with the resolution of chronic HBV infection. Several vaccines have been examined in humans and in animal models, with several tested immunogens, including protein- or peptide-based, DNA- and viral vector-based formulations. Several trials with therapeutic vaccination for chronically HBV infected patients have already been performed[37–40]. From these results, it was clear that the antigen choice was an important immunization strategy and that to obtain immunological success not only HBsAg should be employed. In addition, it was evident that adjuvant options were a critical component to maximize immune HBV responses[41]. Finally, to obtain a total T cell restoration, an accurate patient allocation that could be more likely to benefit from immune modulation represent an essential aspects to consider in the strategy for the new vaccine project.

2.3 Immune Response and Antiviral Control

The liver damage during HBV infection is mainly determined by the host immune response, which often causes the destruction of the infected cells. The virus itself does not appear to manifest a direct cytopathic effect, confirmed also in studies performed

using the HBV mouse model. The relationship between virus and organism appears as a dynamic process within which the virus tries to decrease its visibility and the host tries to prevent and eradicate the infection. This interaction between the virus and the host's immune system is responsible for viral clearance or progression of the infection; in particular the adaptive cell-mediated response seems to play a key role in determining the resolution or, on the contrary, the chronic evolution of the infection[42]. The role of the innate immune response during hepatitis B virus infection is still a matter of debate. The knowledge of innate anti-HBV immune mechanisms is hampered by technical limitations, such as the difficulty of recruiting patients in the pre-symptomatic phase of acute infection and the lack of animal models and cell lines suitable for HBV infection and replication[43]. In vivo studies in chimpanzees and marmots have indicated a weak activation of the intracellular immune response during the early phase of HBV infection, which appears to be weakly perceived by innate immunity. Other studies have shown, on the contrary, the activation of intrahepatic expression of genes involved in the innate immune response immediately after infection in marmots; however, this response appears to be transient and stimulated when inoculated with high concentration of virus[44]. The first events of the innate immune response against the virus, is the release of Type I interferons (IFN-α and IFN-β) from infected hepatocytes. These interferons present a direct antiviral function through the destabilization of the viral capsids, the inhibition of the synthesis of new capsids and the degradation of the preformed HBV RNA. IFN-α and IFN-β also promote the recruitment and activation of antigen-presenting cells such as Kupffer cells (liver-resident macrophages) and dendritic cells. These cells, after being activated, recruit NK cells and macrophages, which play an important role in the initial immune response against the virus[45].

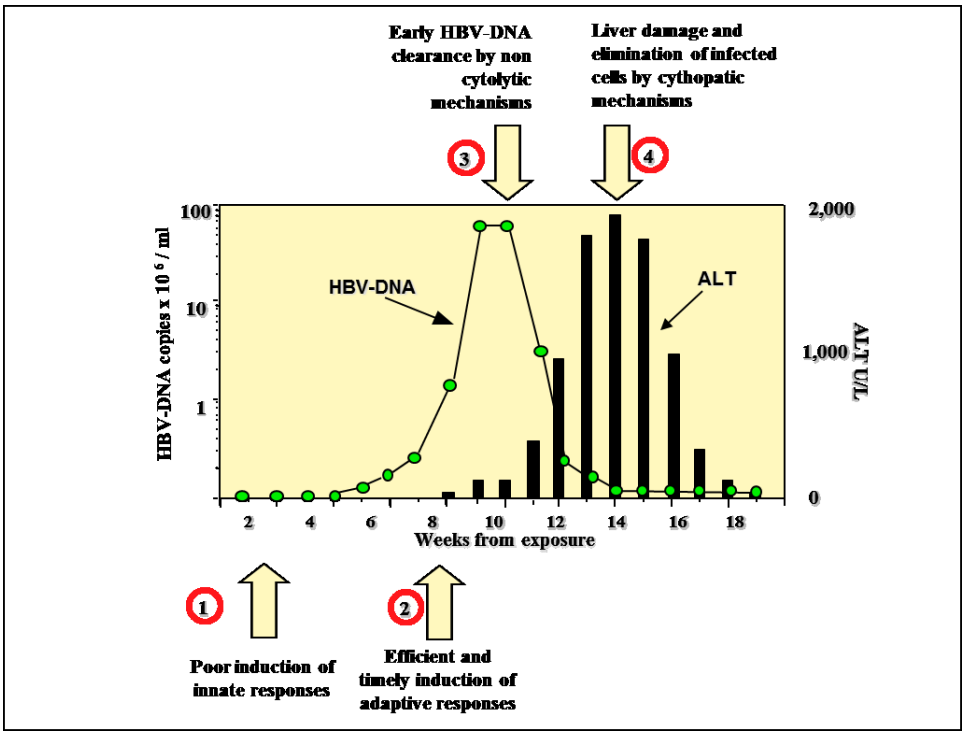

Figure 6. Immunological events after exposure to hepatitis B virus. Early after infection, the innate immune responses has been debated, although seem to be poorly able to "sense" HBV, as shown in the chimpanzee model of infection. After the beginning of rapid viral replication, adaptive responses are timely and efficiently induced. When infection is self-limited, viremia rapidly decreases and most HBV DNA molecules are eliminated from the liver by noncytolytic, cytokine-mediated mechanisms. Cytolytic elimination of the remaining infected cells is finally needed to achieve complete and sustained control of infection. ALT, alanine aminotransferase. Source: Carlo Ferrari, Valeria Barili, Stefania Varchetta and Mario U. Mondelli. Immune mechanisms of viral clearance and disease pathogenesis during viral hepatitis. The Liver: Biology and Pathobiology (in press)

There are many strategies used by HBV to evade this first line of defense implemented by the host organism: the use of a DNA template for transcription (cccDNA) localized in the nucleus of infected cells, the synthesis of a polyadenylated viral mRNA that mimics cellular transcripts, the protection of newly synthesized genomes within viral capsids in the cytoplasm. In addition, HBV appears to be able to block the antiviral activity of Type I interferons and to inhibit the expression of TLRs and the response mediated by these

receptors [43,44]. The humoral immune response also participates in the control of infection, through the synthesis of specific antibodies against viral antigens. HBV-specific antibodies are important since they represent host protection mechanisms as well as they are useful markers for the recognition of different stages of infection. HBcAg-specific IgMs represent an early marker of infection, on the contrary specific antibodies against HBeAg and HBsAg appear later and are indicators of favorable progression of the infection. Anti-HBs antibodies can persist for life in subjects who recover from hepatitis and guarantee a protective immunity against possible reinfection [46]. The antibodies production is a critical element for the neutralization of soluble viral particles; however, it is believed that the evolution of acute infection toward resolution or chronicity depends on the efficiency of the T cell-mediated immune response.

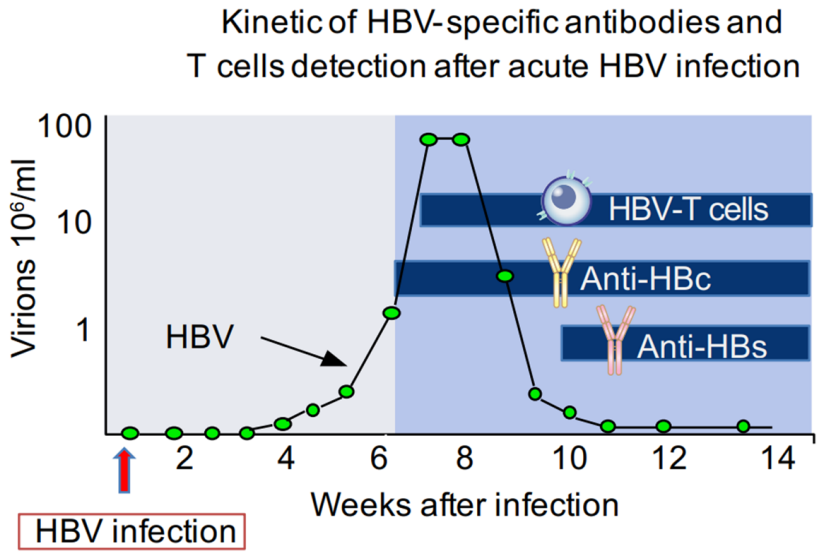

Figure 7. Timing of HBV-specific adaptive immunity. Emergence of HBV-specific antibodies and T cell responses during self-limited HBV infection related to the kinetics of HBV genome replication. Source: Antonio Bertoletti and Carlo Ferrari, Adaptive immunity in HBV infection. J Hepatol. 2016.

Adaptive responses are mediated by CD4+ and CD8+ T lymphocytes, and start about 1-2 weeks after the onset of HBV viral replication within infected hepatocytes (see figure 10). Studies performed on the murine and chimpanzee models have shown that the CD4+ lymphocyte population does not play a central role as effector but is fundamental for the activation and for the maintenance of virus-specific CD8+ T and B lymphocytes[46]. The CD4+ T cells also represent the major IL-2 and IL-21 producers, cytokines that influence the differentiation of CD8 T lymphocytes. CD8+ T cells play a key role during the resolution of HBV infection, in fact, the depletion of these cells in the animal model corresponds to the non-viral clearance. The CD8+ T lymphocytes, as mentioned above, can mediate a cytotoxic mechanism, which promotes direct lysis of infected hepatocytes, and a non-cytotoxic mechanism based on the release of cytokines, such as IFN-γ and TNF-α[47,48]. These cytokines represent mediators for the recruitment of other effector cells, such as macrophages and neutrophils; moreover they can also inhibit viral gene expression within infected hepatocytes without determining cell lysis, thereby counteracting the virus in its intracellular location by limiting hepatic damage[49]. Patients recovering spontaneously from acute HBV infection, typically showed a vigorous and multi-specific CD4+ and CD8+ response, which is capable of recognizing multiple antigenic epitopes of the virus; this guarantees an efficient recognition of infected hepatocytes avoiding to escape from lymphocyte control[50]. However, infection resolution does not correspond to a complete viral eradication, rather it reflects the ability of the immune system, particularly CD4+ and CD8+ T cells, to provide persistent and stringent control over HBV replication. Some HBV-specific T cells remain in an activated state for many years following the resolution of acute hepatitis: these responses seem to be maintained due to continuous stimulation by small amounts of viruses that remain in the host, which

are only detectable through quantitative PCR. This condition of persistence of the virus genome associated with strong suppression of both viral replication and gene expression is termed "occult HBV infection". Conversely, patients with less intense and transient T responses develop chronic HBV infection. In this case the immune system does not seem able to activate a rapid and efficient antiviral response, therefore the virus implements mechanisms to elude the immune control, leading to the persistence of the infection [51,52].

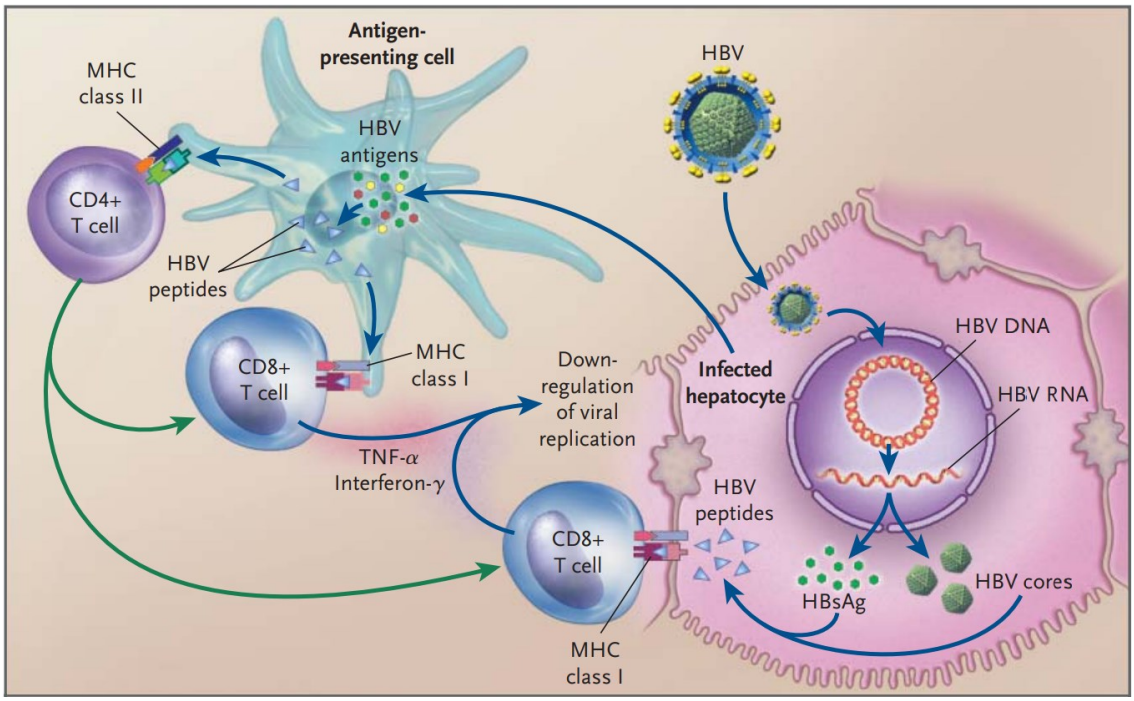

Figure 8. Adaptive Immune Responses to hepatitis B virus. HBsAg particles and virions are produced by replicating HBV in hepatocytes. Both particles have been taken up by antigen presenting cells (APC), to disassemble the viral proteins into peptides to be exposed on the cell membrane together with MHC class I or II complex. These presented antigens can be identified by CD8+ or CD4+ T cells. Antigen-specific CD8+ T cells (with the aid CD4+ lymphocytes, green arrow) can recognize viral peptide on infected hepatocytes. The T-cell recognition drives either direct lysis of the hepatocyte or the IFN-γ and TNF-α secretion, leading to reduction in viral replication in neighboring hepatocytes.
Source: Ganem D, Prince AM. Hepatitis B virus infection--natural history and clinical consequence. New England Journal of Medicine. 2004; 350: 1118-1129.

2.4 T Cell Exhaustion in chronic HBV infection

T-cell *"exhaustion"* is a phenomenon characterized by dysfunction and physical elimination of virus-specific CD8+ T cells that arises during chronic viral infections and cancer. Defective antigen-specific T cells are a key determinant of virus persistence in HBV infection and chronic exposure to high antigen loads is likely involved in altering the regulatory function of co-inhibitory/stimulatory pathways as well as of T cell subsets/cytokines. In fact, full T cell activation depends on both signals released through the T cell receptor and on additional costimulatory signals which may be dysregulated by pathogen replication, contributing to the T cell dysfunction typical of chronic viral hepatitis.

Dysfunctional CD8+ T cells were first discovered during chronic infection in LCMV infected mice, and were also detected in human during infections such as HIV, HCV and HBV as well as in tumors. Specifically, T cell dysfunction develops in a hierarchical manner, in which virus-specific CD8 T cells initially lose IL2 production, proliferative capacity and the cytotoxic activity. Subsequently, early exhausted T cells abrogate the ability to produce TNF-α, and eventually, associated to a severe exhaustion, they lack completely interferon-γ (IFN-γ) production and the capacity to degranulate[53].

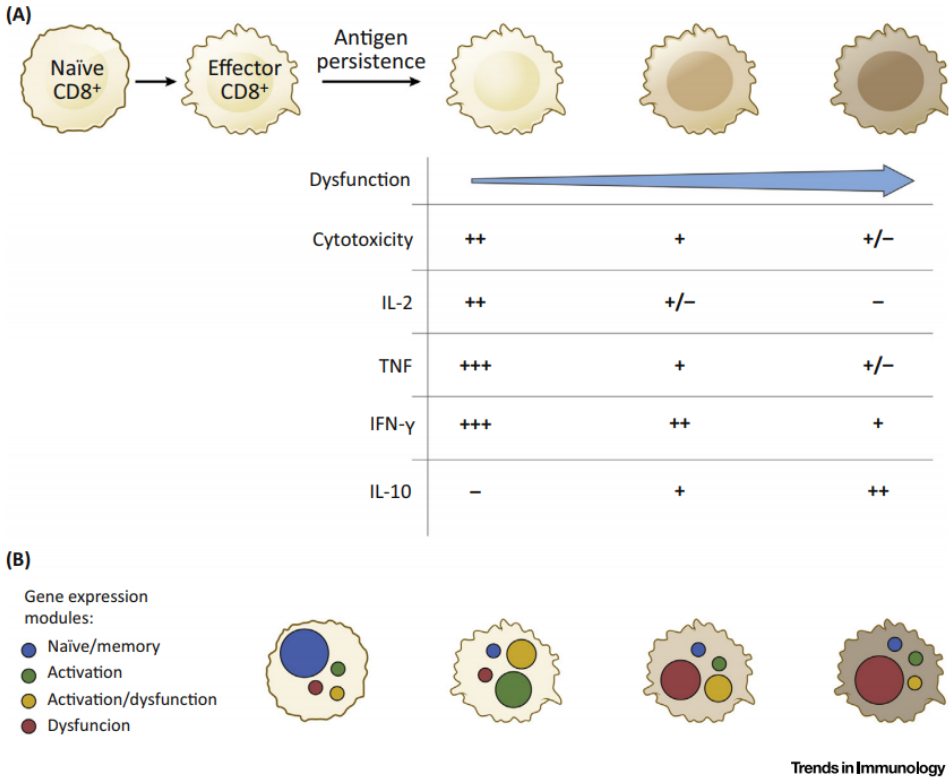

Figure 9. CD8+ T cell phenotypes and gene expression modules in chronic disease. (A) During chronic infection, antigen persistence guides the CD8+ T cell deterioration of effector functions, such as cytotoxicity and cytokine release (IL-2, TNF-α, and IFN-γ). Exhausted T cells can also acquire the capacity to produce immunosuppressive cytokine (IL-10). (B) Naïve/memory, activation, activation/dysfunction, and dysfunction modules related to different CD8+ T cell functional phenotypes. Source: Wang, Singer, Anderson - Molecular Dissection of CD8+ T cell dysfunction. Trends in Immunology 2017

This "*exhaustion*" state defined by poor cytotoxic activity and impaired effector cytokine production is combined to a concomitant expression of multiple inhibitory receptors, such as PD-1, TIM-3, CTLA-4 and CD244. The co-inhibitory PD-1 receptor seems to play a crucial role in the exhaustion phenomenon, being consistently expressed not only in circulating virus-specific lymphocytes of patients with chronic hepatitis, but also in HBV-specific intrahepatic T lymphocytes, which display higher PD-1 expression levels, confirming a more pronounced exhaustion state in the liver[44]. Besides the inhibitory molecules, also some cytokines with suppressive function seem to influence the

exhaustion scenario, such as TGF-β and IL-10, whose expression increases in case of viral persistence contributing to the T lymphocytic dysfunction [54]. The specific molecular signaling underneath T-cell exhaustion remain unclear, several transcriptional pathways have been involved in T-cell dysfunction; e.g., Blimp-1, T-bet and NFAT2 control exhausted T cells during chronic infection, highlighting a distinct lineage fate for the induction of the T cell exhaustion program. Thus, an antigen-mediated transcriptional reprogramming guides the fully exhausted phenotype, typical of dysfunctional T cells, which differs from those of functional effector and memory T cells [54,55].

This is corroborated by recent genomic studies in murine chronic infection, in which the epigenetic profile of exhausted T cells represents a stable differentiation state with an irreversible exhaustion-specific genetic landscape [56,57].

Earlier studies demonstrated that, by blocking PD-1 signaling, T cell functions have been restored in exhausted CD8 T cells, in the context of chronic viral infections. However, regulatory pathway manipulation to improve the functionality of exhausted T cells has been attempted in various experimental settings, and has shown interesting results for T cell recovery; but a full functional restoration has not been achieved so far. Despite the promising results of early-stage clinical trials testing PD-1 signaling blockade as an anti-cancer strategy[58], it is becoming increasingly clear that functional alterations in exhausted CD8 T cells extend beyond inhibitory receptors and immunoregulatory pathways[54]. In chronic HBV infections, inhibitory checkpoints blockade allowed a partial restoration of HBV-specific CD8 T cell functions, with better reinvigoration of intrahepatic than peripheral T cells [59,60] but only a limited proportion of patients are responsive to these in vitro manipulations, indicating the need to identify new molecular targets for T cell function reconstitution strategies.

Indeed, by genome wide transcriptional profiling of HBV-specific CD8+ cells from chronic patients, down-regulated genes coding for different mitochondrial, proteasomal, DNA repair and transcriptional regulation components were identified [61]. These results highlight the importance of metabolic defects - in dysfunctional T cells - which are associated to a poor mitochondrial capacity for the cellular energy demands, and which have been confirmed by the response to mitochondria-targeting antioxidants and natural polyphenols treatments that improved *in vitro* function and viability of exhausted T cells[62,63].

For the design of efficient immunotherapies for chronic HBV infection, a better understanding of HBV-specific CD8+ T cell biology, especially of the dominant antigen targets and their distinct phenotypic, metabolic and functional profile, is required.

In-depth analyses of virus-specific lymphocytes at the single cell level in chronic infections, displayed that fully exhausted CD8 T cells are not a homogeneously inefficient population, but co-exist subsets of dysfunctional cells with variable levels of co-inhibitory molecules, transcription factors, marker of terminal differentiation, chemokines and other receptors [59,60,64–66].

Exhausted HBV-specific T cells up-regulate also the death receptor TRAIL-2 and the pro-apoptotic mediator BIM, that may contribute to the deletion of HBV-specific CD8 T cells [67–69]. Further features of exhausted T cells are the loss of memory markers (e.g. CD127, CD44, CD62L or CXCR3), the poor response to IL-7 and IL-15 needed for the homeostatic maintenance of memory cells and the up-regulation of the ectonucleotidase CD39[70], prostaglandin E2 receptors[71], as well as transcription factors such as Eomesodermin (Eomes) and Blimp-1[59,64].

According to the differential expression of the T-box transcription factors Tbet and Eomes in conjunction with PD-1, two distinct subsets of LCMV-specific CD8 cells have been reported in chronically LCMV infected mice which exhibited impaired function but different effector activity[59]. The $Tbet^{high}Eomes^{dim}PD\text{-}1^{int}$ subset represents a smaller progenitor pool with residual proliferative potential, while the $Tbet^{dim}Eomes^{high}PD\text{-}1^{high}$ subset is a numerically larger population of exhausted T cells with higher expression of PD-1 and other inhibitory receptors, and limited proliferative capacity[72]. Persistence of antigen causes $Tbet^{high}PD\text{-}1^{int}$ cells to lose Tbet expression[73], to undergo proliferation and to convert into $Tbet^{dim}Eomes^{high}PD\text{-}1^{high}$ cells with weaker cytokine production and very poor response to PD-1 pathway blockade, which can instead reinvigorate more efficiently the $Tbet^{high}Eomes^{dim}PD\text{-}1^{int}$ subset[59]. Further dissection of the virus-specific CD8 T cell pool into functionally distinct subsets has been reported in different models of chronic viral infections by looking at the co-expression of the chemokine receptor CXCR5[74], the transcription factor TCF-1, CD127 together with PD-1[60].

Recent studies demonstrate a key role for the HMG-box transcription factor (TOX) in the induction of T cell exhaustion. TOX directly regulates the epigenetic landscape of dysfunctional T cells, which could represent an important marker for the comprehension of the T cell deregulation reversibility as well as a crucial target for the re-invigoration immunotherapies[75]. In fact, deletion of Tox DNA-binding domain, in the LCMV mouse model, diminishes the mRNA and protein PD-1 levels, resulting in increased cytokine production and more polyfunctional T cells[76]. Tox displays a high expression level in chronic HCV infected antigen-specific lymphocytes, with a reduced expression after therapy and a complete absence in those patients that spontaneously resolved;

confirming again the direct role of the Tox transcription factor in the different stages of infection[76].

Furthermore, in chronic HCV infection, a stem cell-like progenitor subset of HCV-specific T cells, expressing the CD127 T-cell memory marker and the co-receptor PD-1 ($CD127^{+}PD1^{+}$), is maintained after the infection resolution and this distinctive subset retains the proliferative capacity (mediated by the transcription factor TCF1), unlike the defective and terminally exhausted $TCF1^{-}CD127^{-}PD^{1high}$ subset [65].

More limited information is available regarding virus-specific CD8 T cell subsets in HBV infection where spontaneous virus control is strongly correlated with high Tbet expression, and where chronic phase is instead associated to lower expression[61,66].

Phenotypic investigation of the virus-specific T cell populations identifies peculiar differences based on the targeted HBV protein. In particular, envelope-specific T cells show high $PD\text{-}1^{high}$ expression during acute infection, which is associated to a lower expression of those targeting both core- and polymerase-epitopes[77]. This epitope-specific hierarchy of PD-1 levels is preserved even in the absence of viremia or circulating HBV particles in acute HBV infection[77]. In chronically infected host (with high and low viral loads), envelope-specific T cells is not detectable and those targeting core protein, express higher level of PD-1 with respect to polymerase-specific CD8 cells. Significant differences are evident also between core and pol epitope, such as T cell differentiation markers, T cell functions and T cell regulatory phenotypes[77]. In particular, pol-specific CD8+ T cells exhibit a naive-like phenotype in CHB patients, probably for an abortive activation or an inefficient recruitment to the effector T cell pool supported by the reduced *in vivo* frequencies [78]. In this view, the antigen levels presented on hepatocytes (such as the high levels of core protein), may be correlated to T cell frequencies and

function [78]. Core-specific CD8+ T cells display also a better expansion capacity in vitro [78], irrespective to the PD-1 expression level, and those specific for the polymerase epitope express higher level of KLRG1 and Eomes as compared to those targeting core, together with low level of Tbet [78]. Furthermore, core-specific CD8+ T cells show a higher frequency of the memory-like subset (CD127+PD1+), which correlates with the proliferative potential *in vitro*. This correlation was not relevant for the pol T cell pool, even though both pol- and core-specific CD8 T cells express an equal level of the TCF1 transcription factor [78]. Rather, an important anti-apoptotic marker (Bcl-2) correlates with this different expansion capacity of the two T cell pools, confirming the relevance to investigate better this molecular marker [78].

These findings highlight clearly the existence of distinct molecular 'fingerprints' of T cell sub-populations associated to antigen epitopes and also to the infection stage, whose molecular dissection represented the starting point for more focused and personalized new immunotherapies.

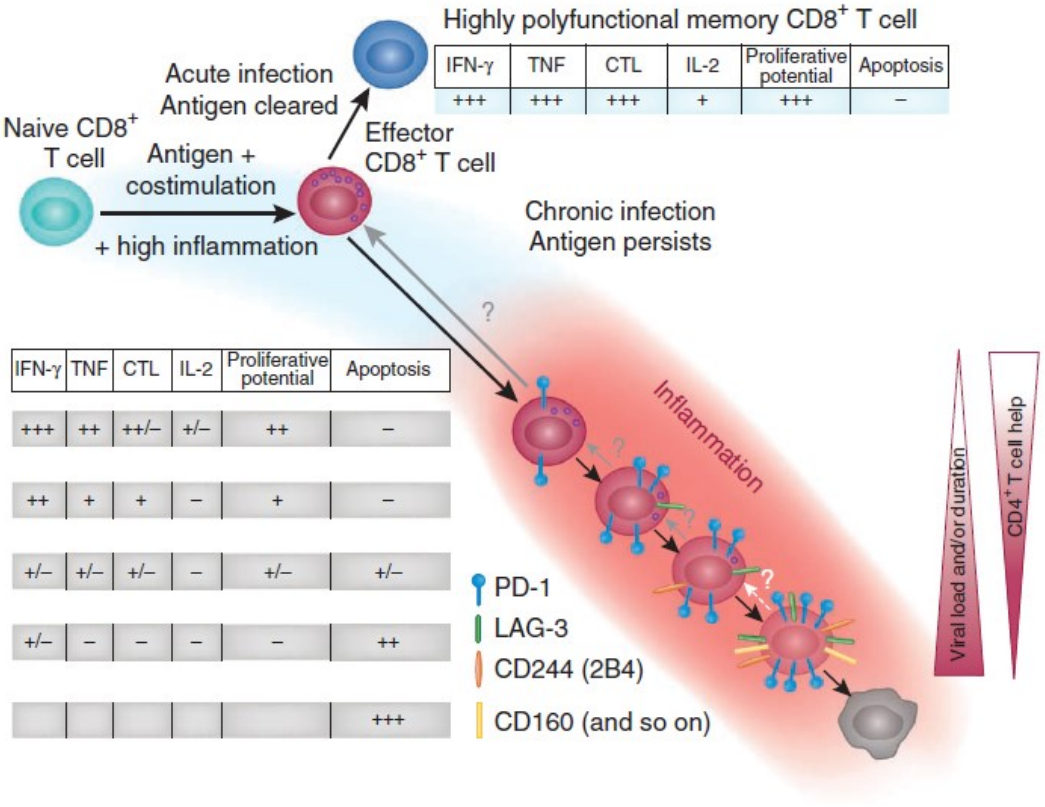

Figure 10. T cell exhaustion in chronic disease. During early phase of infection, naive T cells are stimulated by antigen, co-stimulation and inflammation and develop into effectors. During self-limited acute infection functional effector T cells generate memory T cells (highly polyfunctional) which release many cytokines (such as IFN-γ, TNF-α and IL-2), are cytotoxic (CTL) and have a vigorous proliferative capacity (top). During chronic infection (bottom), the virus persists. As viral load increases, T cells become progressively dysfunctional, missing effector T cell functions in a hierarchical way, acquiring high expression of several inhibitory receptors. Finally, antigen-specific T cells can undergo to apoptosis with complete elimination of virus-specific T cell responses. Correlation between T cell exhaustion, high viral load, absence of CD4+ T cell function and prolonged infection has been shown on the right. The intensity of each property is expressed on a scale format from high (+++) to low (—). Source: Wherry EJ. T cell exhaustion - Nature Immunology, 2011.

BACKGROUND AND AIM OF THE STUDY

An urgent clinical need in HBV infection is to develop novel therapies for chronic hepatitis. Since HBV-specific T cells play a crucial role in antiviral protection and failure to control HBV is associated with a deep impairment of T cell responses, a possible therapeutic strategy is to correct HBV-specific T cell exhaustion[54,55]. Exhausted virus-specific CD8+ T cells exhibit a high expression of the co-inhibitory receptor PD-1 and other inhibitory checkpoints, including 2B4, CTLA-4, Tim-3, Lag-3, TIGIT, BTLA, CD160[48] and PSGL1[55]. High PD1 expression is detectable in over 90% of liver-infiltrating HBV-specific CD8 T cells in chronic HBV infection, while 2B4, LAG3 and CD160 are less expressed within the liver [59,79,80]. Further features of exhausted HBV-specific T cells are the up-regulation of the death receptor TRAIL-2, the pro-apoptotic mediator BIM[60,65,66] , the ectonucleotidase CD39[70] and prostaglandin E2 receptors[71], together with the loss of memory markers (e.g. CD127, CD44, or CD62L).

Available data indicate that exhausted virus-specific CD8 T cells are a heterogeneous dysfunctional population. Indeed, different studies of CD8 T cell exhaustion in the LCMV model of chronic virus infection and also in human chronic HCV infection indicate that simultaneous detection of different transcription factors, co-inhibitory receptors and differentiation molecules can allow to distinguish T cell subsets with different antiviral activity and different responses to checkpoint blockade, from more terminally exhausted T cell subsets with higher expression of PD-1 and other inhibitory receptors, limited antiviral activity and less responsive to PD-1 pathway blockade, to more memory-oriented subsets endowed with lower PD-1 expression and better functional capabilities[57]. In chronic HCV infection both $TCF1^{-}CD127^{-}PD\text{-}1^{hi}$ terminally exhausted and

TCF1^{+}CD127^{+}PD-1^{+} memory-like T cell subsets have been identified. Importantly, the memory-like subset with increased functionality was maintained after antiviral treatment and HCV elimination, without reaching the functional efficiency of the real memory T cells generated after spontaneous control of acute HCV infection[65].

Limited information is available about subsets of virus-specific CD8 T cells in HBV infection where spontaneous virus control has been reported to be strongly associated with high Tbet expression that was instead impaired in dysfunctional CD8 cells from chronic patients[66]. Moreover, the HBV-specific CD8 T cell pool includes subsets of liver-resident memory T cells (TRM), which display protective potential, with high IL2 production allowing long-term survival and local anti-viral activity[81].

Recently, novel information about the heterogeneity of exhausted virus-specific CD8 T cells was reported also during chronic HBV infection. Even though the HBV-specific T cell population appears globally dysfunctional, in chronically infected patients with low levels of HBV-DNA polymerase-specific CD8 T cells were reported to express a higher degree of T cell exhaustion with up-regulation of CD38, KLRG1 and Eomes compared to core-specific CD8 T cells. This latter population showed an enhanced expansion capacity associated with a more protective memory-like CD127^{+}PD1^{+} phenotipic profile, highlighting the co-existence in these patients of CD8 T cell populations targeting different epitopes with distinct phenotypes and functions[78]. Moreover, the relative contribution of different T cell specificities to HBV control were recently studied also in the setting of acute self-limited hepatitis B and in patients with eAg-negative CHB characterized by heterogeneous levels of viral and antigen loads[77]. CD8 T cells targeting distinct HBV proteins/epitopes displayed different phenotypic and functional features at both stages of infection with envelope responses particularly impaired in CHB patients. A

hierarchy of PD-1 expression associated with responses towards different HBV specificities was mantained in self-limiting hepatitis B subjects after control of infection and also in chronic patients, with core-specific CD8 T cells expressing higher level of PD-1 compared to CD8 cells targeting the polymerase protein. These different T cell phenotypes also translated into distinct functional capacities with lower levels of IFN-γ secretion and CD107 degranulation by polymerase-specific T lymphocytes in comparison with core-specific T cells[77].

Based on these premises, aim of the study was to define how heterogeneous are exhausted HBV-specific CD8 cells in HBV infection, how the relative representation of different phenotypic profiles T cell subsets in individual patients can reflect the overal exhaustion condition of individual patient T cells and and whether T cell subset analysis can be helpful to predict response to immune modulators in view of the possibe design of personalized immune therapeutic strategies. To address these issues, HBV-specific CD8 T cells were analyzed *ex vivo* for cytokine production and for different phenotypic checkpoints/differentiation markers in eAg-negative chronic naive patients with high viral load and fluctuating ALT levels. The study was also extended to chronic NUC-treated patients with complete control of infection and to spontaneous controllers, as reference patient groups able to obtain viral control and develop anti-HBs antibodies following long-term chronic carriage of the virus.

MATERIALS AND METHODS

1. PATIENT COHORTS

The study was conducted at the U.O. of Infectious Diseases and Hepatology of the Parma University Hospital. The experimental protocol and the informed consent form were examined and approved by the local Ethics Committee. Any study-related activity was undertaken only after obtaining and collecting informed consent of each patient. Patients with HLA-A2 positive profile are poorly represented in human population, therefore more that one hundred chronic HBV infected patients were examined to reach an adequate samples number and get solid statistical values.

Only patients with a well characterized prior history of disease or infection will be recruited.

1. 18 Chronic naive viremic patients (total lack of virus control: HBsAg positive, anti-HBc positive, HBeAg negative/anti-HBe positive, serum HBV-DNA positive with viremia levels >20,000 IU/ml and elevated alanine aminotransferase (ALT).
2. 11 immune subjects (complete viral control: HBsAg negative, anti-HBs positive, anti-HBc positive, serum HBV-DNA negative) as a conseguence of spontaneous HBsAg clearance following a long time of chronic carriage of the virus (either active or inactive)
3. 8 immune subjects (complete viral control: HBsAg negative, anti-HBs positive, anti-HBc positive, serum HBV-DNA negative) as a conseguence of HBsAg clearance following a long-term NUC therapy

All patients were negative for anti-hepatitis C virus, delta virus, human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2) antibodies and for other markers of viral or autoimmune hepatitis.

To select patients with HLA-A2 profiles, staining with the fluorescent antibody anti-HLA-A2.01 (BD Biosciences, San Jose, CA) was performed on PBMCs and subsequent assessed by flow cytometry analysis.

		GENDER	GENOTIPO	HBsAg (UI/ml)	anti HBs	HBeAg	ALT (GPT)	HBV-DNA
CHB	1	M	D	3560	-	-	31	185600
	2	M	D	4085	-	-	85	3497520
	3	M	n.d.	1932	-	-	136	289
	4	M	D	2752	-	-	40	47**
	5	F	D	482	-	-	43	66400
	6	F	n.d.	5398	-	-	30	35801
	7	F	A	4600	-	-	65	229402
	8	M	D	n.d.	-	-	165	260000
	9	M	D	608	-	-	93	881000
	10	M	D	694	-	-	49	9856
	11	M	n.d.	n.d.	-	+	49	20700
	12	M	n.d.	4775	-	-	68	153246
	13	F	n.d.	714	-	-	30	11453
	14	F	D	19679	-	-	34	509000
	15	F	C	144	-	+	51	6.800.000
	16	M	n.d.	2662	-	-	40	21331
	17	M	n.d.	3736	-	-	146	1400591
	18	M	n.d.	3472	-	-	121	8132680
NUC resolved	19	M	n.d.	-	33	-	14	0
	20	M	D	-	26	-	28	0
	21	M	D	-	190	-	19	0
	22	F	F	-	173	-	10	0
	23	M	A	-	325	-	23	0
	24	M	D	-	4	-	19	0
	25	M	A	-	506	-	17	0
	26	M	n.d.	-	2	-	29	0
Spontaneus Seroconversion	27	M	n.d.	-	45	-	28	0
	28	F	n.d.	-	66	-	39	0
	29	M	n.d.	-	n.d.	-	22	0
	30	M	n.d.	-	270	-	29	0
	31	F	n.d.	-	7	-	15	0
	32	F	n.d.	-	451	-	11	0
	33	M	n.d.	-	+	-	20	0
	34	M	n.d.	-	-	-	24	0
	35	M	n.d.	-	3	-	15	0
	36	M	n.d.	-	59	-	12	0
	37	F	n.d.	-	n.d.	-	14	0

Table 2. Clinical details of patients with chronic HBV hepatitis.

2. IMMUNOLOGICAL ANALYSIS

2.1 PBMC isolation

Peripheral Blood Mononuclear Cells (PBMCs) were obtained from fresh heparinized blood by Lymphosep (Biowest) density gradient centrifugation. Briefly, 20ml of heparinized blood was stratified on 13ml of Lymphosep and centrifuged at 2200 rpm for 20 minutes. The PBMCs ring, formed at the interface between Lymphosep and plasma, was then collected and washed twice with Hank's saline solution. The count of the recovered cells was performed by staining with Türk using the Neubauer chamber. The PBMCs were then frozen in freezing medium (90% of Bovine Fetal Serum and 10% of Dimethylsulpoxide, DMSO) and cryopreserved in liquid nitrogen until use.

PBMCs were thawed in complete medium (RPMI 1640 with 8% human serum, 1% penicillin-streptomycin) and incubated for 15–30 min at 37 °C in complete medium before processing.

2.2 Peptides and dextramers

To evaluate virus-specific CD8 T cells response in HLA-A2 positive patients, peptides covering the HLA-A2-restricted epitopes of the core (aa 18–27: FLPSDFFPSV), envelope (aa 335-343: WLSLLVPFV) and polymerase (aa 455–463: GLSRYVARL) of HBV genotype D were used. As control, peptide corresponding to HLA-A2-restricted epitope of influenza A virus (FLU) matrix (GILGFVFTL), was employed. Peptides were purchased from Proimmune (Oxford, UK).

The PE- and APC- labeled dextramer peptide-HLA class I complexes corresponding to HBV-core, HBV-envelope, HBV-polymerase and FLU matrix were purchased from Immudex (Copenhagen, Denmark).

2.4 Phenotypic analysis of HBV-specific CD8+ cells.

To define the stage of CD8 T cell differentiation, dextramer positive CD8+ cells were evaluated for *ex-vivo* expression of cytokine receptors (CD127) and anti-apoptosis/cell survival biomarkers (Bcl-2 and TCF-1). Expression of negative costimulatory molecules such as PD-1, TOX and CD39, utilized as markers of T cell exhaustion, were also tested.

Once thawed, the PBMCs were suspended in RPMI-1640 containing 8% human serum and incubated with monoclonal antibodies: specific dextramer-PE, CD3-BV510 (Becton Dickinson), CD39-APC-Vio770 (Miltenyi), CD127-PE-CF594 (Becton Dickinson), PD1-PeCy7 (Invitrogen) and CD8-BV786 (Becton Dickinson).

After washing with PBS 1X, the cells were fixed for 30 minutes at 4°C with Fixation Permeabilization Concentrate and Diluent (eBioscience) then washed twice with Perm/Wash BD Trascription Factor (BD) following the manufacturer's instructions. Finally, cells were stained for 45 minutes at 4 °C with monoclonal antibodies TCF1-Alexa488 (Cell Signaling Technology), Tox-APC (Miltenyi), and Bcl-2-BV450 (Becton Dickinson). The samples were washed twice with Perm/Wash BD Trascription Factor (BD) before flow cytometry acquisition.

All determinations were performed using an 8-fluorescence flow cytometer (FACSCANTO II, Becton Dickinson, BD, Immunocytometry System, CA, USA) and an 18-fluorescence flow cytometer (LRSFortessa, BD). The data were processed with the FACS-DIVA software or Flow-Jo software (BD), identifying virus-specific CD8 T cell subsets based on the CD127 and PD-1 expression.

Subsequently, these subpopulations were classified, evaluating the co-expression with TOX, CD39, Bcl-2 and TCF-1. Data were expressed as Median Fluorescence Intensity (MdFI) for TOX, Bcl-2 and TCF-1 and as percentage for the exhaustion marker CD39.

2.3 Functional assessment of HBV-specific T cells.

Since HBV-specific T cells in CHB patients are generally hyporesponsive *ex vivo* to peptide stimulation, several preliminary experiments were performed applying stimuli acting at different levels of the T cell signaling cascade (anti-CD3/anti-CD28, PMA/ionomycin, IL2, IL12) in order to further dissect the *ex vivo* T cell impairment. By these analysis, PMA/Ionomycin stimulation was selected for *ex vivo* cytokine evaluation.

The detection of intracellular cytokines (TNF-α and IFN-γ) production (Intracellular Cytokine Staining, ICS) was performed on dextramer positive CD8+ following ex vivo stimulation with PMA (phorbol 12-myristate 13- acetate) (100ng/ml) and ionomycin (1µg/ml) for 4 hours. PMA is a small organic compound which diffuses through the cell membrane into the cytoplasm, where it directly activates Protein Kinase C (PKC), while the Ionomycin is a calcium ionophor that is used to raise intracellular calcium levels. After one hour of stimulus, Brefeldin A (BFA, 10 µg/ml, BD) was added. BFA inhibits protein transport from the Golgi apparatus to the endoplasmatic reticulum, and prevents the secretion of neo-synthesized cytokines outside the cell. Incubation was continued for another 3 hours and the cytokines thus accumulated in the cell cytoplasm were detected in flow cytometry. After the 4 hours of incubation, the cells were first washed and then stained with surface antibodies: dextramer-PE (Immudex), CD8-APC-H7 (Becton Dickinson), CD3-PerCp (Miltenyi), CD39-VioBright (Miltenyi), CD127-PE-CF594 (Becton Dickinson), PD1-PECy7 (Invitrogen)) for 15 minutes at RT, then fixed for 30 minutes at 4°C with Fixation Permeabilization Concentrate and Diluent (eBioscience), then washed twice with Permeabilization Buffer (eBioscience) following the manufacturer's instructions. Finally, cells were stained for 30 minutes at 4 °C with monoclonal antibodies TNF-α-APC and IFN-γ-APC-R700 (Becton Dickinson). The samples were washed twice with

Permeabilization Buffer before acquisition. Cells were acquired on a BD LSRFortessa multicolor flow cytometer and were analyzed with the FACSDIVA and FlowJo software (Becton Dickinson). Data were expressed as total cytokine frequency that described the total amount of TNF-α single positive, IFN-γ single positive and TNF-α IFN-γ double positive in the dextramer-specific CD8 T cells.

2.4 Definition of "*Exhaustion Index*" and "*Functional Index*".

Based on the exhaustion and memory markers expression, PD1 and CD127 respectively, HBV-specific CD8 T cells were characterized by flow cytometry. The ratio between the percentage of $PD1^{high}$ $CD127^{low}$ and $PD1^{low/-}$ $CD127^{high}$ HBV-specific CD8 cells, defined *Exhaustion Index*, was calculated for each patient.

Functional Index was obtained for each patients as the ratio between the percentage of total cytokine production in HBV-specific CD8 cells and the percentage of double negative cells among the same population.

2.5 Statistical methods.

The GraphPad Prism software was used for statistical analysis. Before every comparison, normality distribution of data was tested by the Kolmogorov–Smirnov test, and nonparametric statistic was applied. The F test of variance was applied to the different groups. Comparisons were done by the Mann-Whitney U test and the Wilcoxon-matched-paired test. A p value <0.05 (two tailed) was considered significant. Data correlations will be evaluated by Pearson and Spearman tests.

RESULTS

Phenotypical analysis of HBV-specific CD8 T cells suggests T cell exhaustion heterogeneity in chronically HBV-infected patients.

HBV-specific CD8+ T cells targeting HLA-A*02-restricted epitopes, $core_{18}$, pol_{455}, and env_{183}, were analyzed *ex vivo* in 18 HBeAg- chronic naive patients with high viral load and fluctuating ALT levels, 10 chronic NUC treated patients with complete control of infection (anti-HBs+) and 10 spontaneous controllers following long-time chronic carriage of the virus. By using a dextramer-based approach, among the different HLA-A2 restricted specificities, $Core_{18\text{-}27}$ specific CD8 T cells were the only T cell population detectable in the majority of chronic HBV-infected patients. Since Pol_{455} and Env_{335}-specific CD8+ T cells were only found in HBV resolvers, phenotypic comparison among different patient categories was focused on $Core_{18}$-specific CD8+ T cells. In line with previous studies reporting very low frequencies of HBV-specific T cells in the peripheral blood of HLA-A*02 chronic viremic patients, more that one hundred patients had to be examined to reach the number of patients planned for the study with a detectable frequency of HBV-specific CD8 T cells to allow their phenotypic analysis.

CD8 T cells specific for Core 18-27 were characterized *ex vivo* by flow cytometry for the co-expression of the exhaustion and memory markers PD1, CD39, TOX, CD127, Bcl-2 and TCF1. To get a first discrimination of different exhaustion T cell subsets, phenotypic analysis was initially based on PD1 and CD127 co-expression on HBV-specific dextramer+ CD8 T cells. Importantly, a subset of $PD1^{high}$ $CD127^{low/-}$ (defined as subset 4, red box in figure 11 A) was selectively prevalent in naïve viremic patients (red dots and bars in figure 11 B-C respectively). Conversely, the phenotypic profile of Flu-specific CD8 cells derived

from the same patients population, was completely dominated by PD1$^{low/-}$ CD127^{high} cells (defined subset 1, green box and dots in figure 11 B), expected to be more protective, without representation of the PD1^{high} CD127^{low} subset deteced in chronic patients. Instead, the more protective PD1$^{low/-}$ CD127^{high} profile was preferentially represented among HBV-specific CD8 T cells in controllers but only in a few chronic naïve patients. Finally, a PD1^{high} CD127^{high} HBV-specific CD8 T cell subset(defined subset 2, blue box in figure 11 A) was present in all groups of patients, irrespective of the clinical condition and level of virus control (blue dots and blue bars in figure 11 B-C respectively).

Based on the assumption that the prevalence of the PD1$^{low/-}$ CD127^{high} over the PD1^{high} CD127^{low} HBV-specific CD8 T cell subset in individual patients should be indication of better anti-HBV protection, the ratio between the percentage of the two HBV-specific CD8 T cell subsets was calculated, as a numerical parameter, named *Exhaustion Index,* to select the patients with different degrees of exhaustion (figure 12 B).

Interestingly, by this approach, 6 chronic patients showed values of exhaustion index close to 0 and to the median value of those calculated for NUC and spontaneous resolvers able to develop anti-HBs antibodies (figure 12).

In summary, these data demonstrate that in chronic active HBV infection exhausted T cells are a heterogeneous cell population with variable phenotype. Phenotypic profiling based on the quantification of PD1 and CD127 expression on HBV-specific CD8 T cells may allow to predict different levels of T cell exhaustion at individual patient level suggesting the potential predictive value of this *Exhaustion index.* However, it is important to note that, although control of infection in NUC and spontaneous resolvers enables HBV-specific CD8 cells to acquire a less exhausted phenotype, this phenotypic profile does not become identical to that detected in flu-specific CD8 T cells.

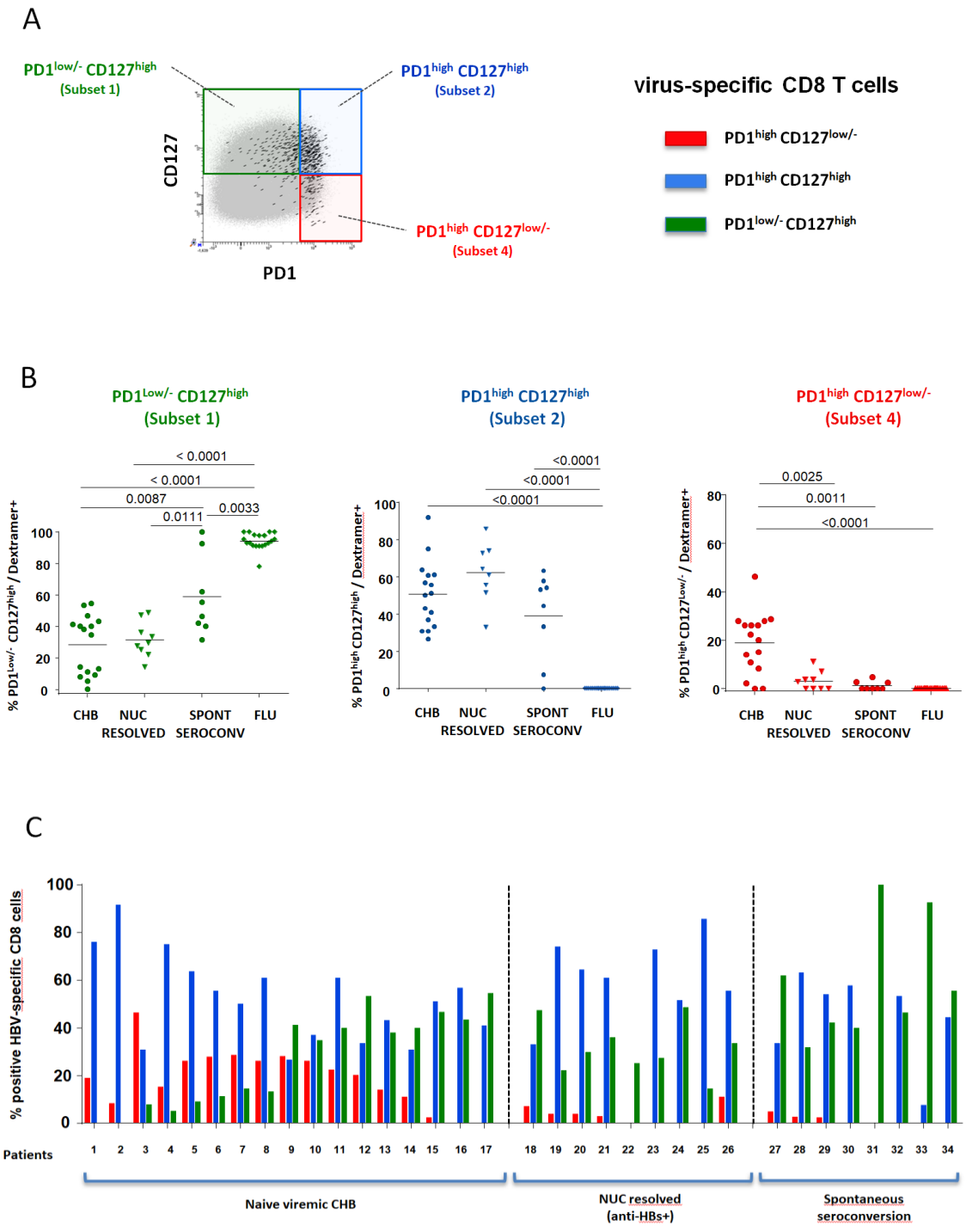

Figure 11. Representation of the different CD8 T cell subsets in chronic viremic naive compared to HBV resolver patients (anti-HBs+ NUC treated patients and spontaneous seroconversions).

A) PD1 and CD127 co-expression allows to distinguish 3 differentHBV-specific CD8 T cellsubsets: PD1$^{low/neg}$ CD127^{high} (subset 1, green box), PD1^{high} CD127^{high} (subset 2, blu box) and PD1^{high} CD127$^{low/neg}$ (subset 4, red box). A representative FACS plot in a naïve chronic patient is illustrated. B-C) Frequencies of the different HBV-specific CD8 T cell subpopulations are represented as dots (B) and bars (C) in the different patient categories. Statistically significant differences between different patient categories detected by the Mann-Whitney test are indicated

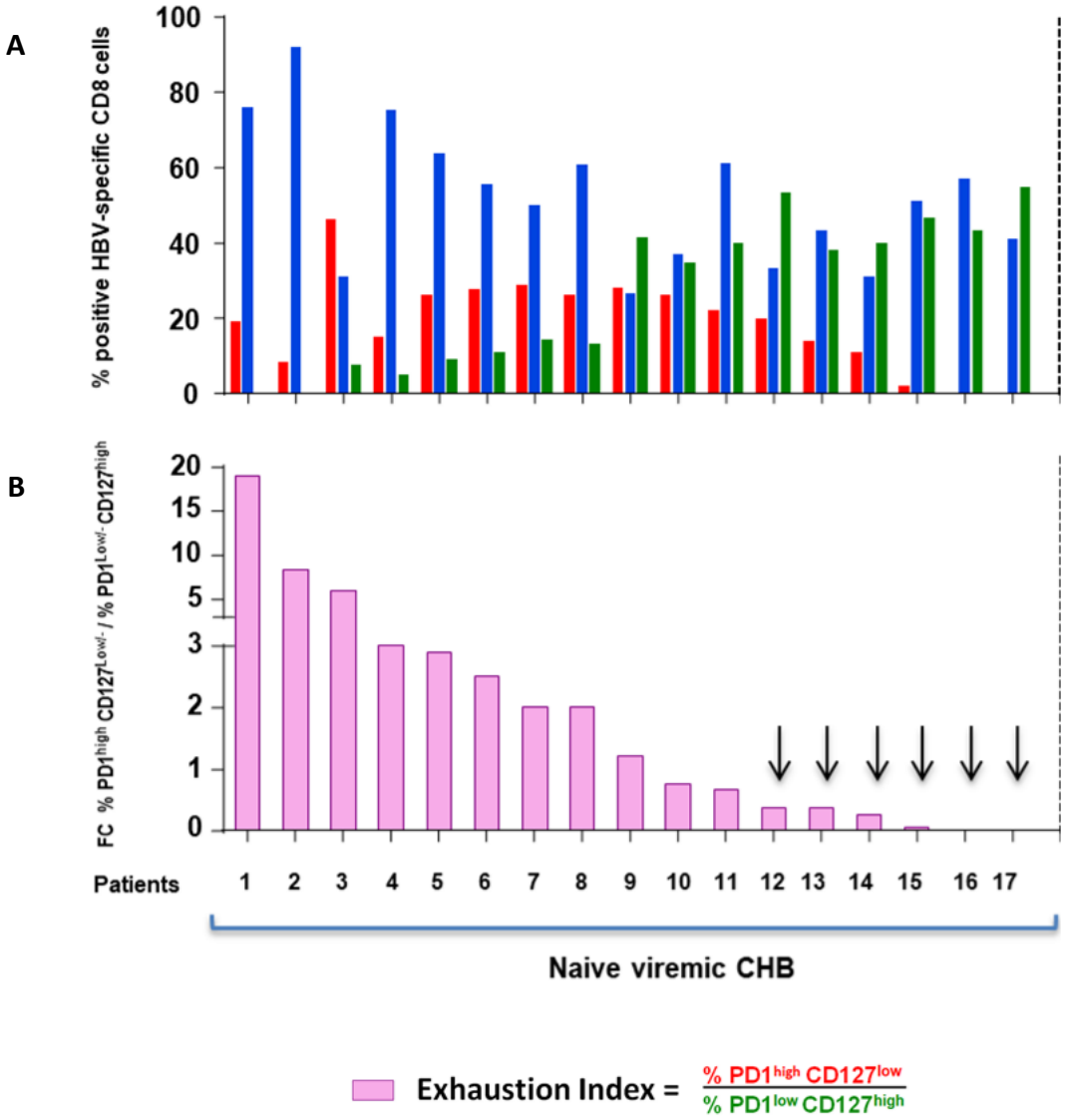

Figure 12. Relative representation of PD1^{high} CD127^{low} versus PD1$^{low/-}$ CD127^{high} subsets to predict different levels of T cell exhaustion in naive viremic CHB. A) Frequencies of the different HBV-specific CD8 T cell subpopulations, as definedby PD1 and CD127 expression levels, are represented as bars in naive viremic CHB. B) Ratio between the percentage of PD1^{high} CD127^{low} and PD1$^{low/-}$ CD127^{high} cells is defined *Exhaustion Index* (pink bars). Chronic patients indicated by the arrows show values of exhaustion index close to 0 and very similar to those calculated for NUC and spontaneous resolvers able to develop anti-HBs antibodies.

Ex vivo functional characterization of HBV-specific CD8 T cell subsets. Next, to further investigate the functional profiling of the different exhausted HBV-specific T cell populations in patients with chronic HBV infection, $core_{18}$-specific CD8 T cells were analyzed *ex vivo* after 4 hours PBMC stimulation with PMA and Ionomycin by intracellular cytokine staining for the expression of IFN-γ and TNF-α (figure 13). Since HBV-specific T cells in CHB patients are generally hyporesponsive *ex vivo* to peptide stimulation, preliminary experiments were performed applying stimuli acting at different levels of the T cell signaling cascade (anti-CD3/anti-CD28, PMA/ionomycin, IL2, IL12) in order to identify the best experimental conditions for T cell stimulation. These experiments revealed that upon PMA/ionomycin stimulation variable intensity of HBV-specific T cell cytokine production could be observed.

Analyzing the total percentage of $core_{18}$-specific CD8 T cells able to produce cytokines in the different patient categories, naive viremic CHB patients showed lower levels of IFNγ and TNFα production *ex vivo* compared to resolved patients (NUC-resolved and spontaneous controllers) and to flu-specific CD8 T cells derived from each individual patient (figure 13 C). A similar difference was also detected by the analysis of double positive (IFNγ+TNFα+) (data not shown) and double negative (IFNγ-TNFα-) dextramer-specific CD8 T cells (figure 13 D).

Moreover, the functional analysis of the different CD8 T cell subsets revealed variable levels of cytokine production *ex vivo*. Notably, $PD1^{high}$ $CD127^{low}$ cells were less efficient than $PD1^{low}$ $CD127^{high}$ cells in cytokine production (figure 14 A).

Cytokine production by individual T cell subsets was then compared between the different patient populations. The $PD1^{low}$ $CD127^{high}$ cell subset showed similar levels of cytokine production in all study categories, namely naive viremic CHB, NUC resolved

patients and spontaneous controllers (figure 14 B). The $PD1^{high}$ $CD127^{high}$ cell subset was heterogeneous in naive viremic CHB patients and generally less efficient in cytokine production than in NUC and spontaneous resolvers able to develop anti-HBs antibodies (figure 15 A). A similar comparison could not be done for the $PD1^{high}$ $CD127^{low}$ T cell subset because it was poorly or not at all represented among NUC and spontaneous resolvers.

Importantly, looking at the correlation between cytokine production by the $PD1^{high}$ $CD127^{high}$ cells subset and the values of the *Exhaustion Index*, as defined above by phenotypic parameters, naive viremic CHB patients with a low EI (blue dots and blue background, figure 15 B-C) were those with maximal cytokine production, comparable to resolver patients. Instead, chronic patients with high EI (orange dots and orange background, figure 15 B-C) showed significantly lower cytokine production compared to chronic patients with high EI and to resolvers. The correlation between phenotypic and functional parameters was less stringent when values of *Exhaustion Index* were assessed in relation to cytokine production by the overall HBV-specific CD8 T cell population instead of the $PD1^{high}$ $CD127^{high}$ cells subset (data not shown).

Thus, calculation of the *Exhaustion Index* based on phenotypic parameters can allow to identify two groups of CHB patients with different functional profiling and different levels of exhaustion.

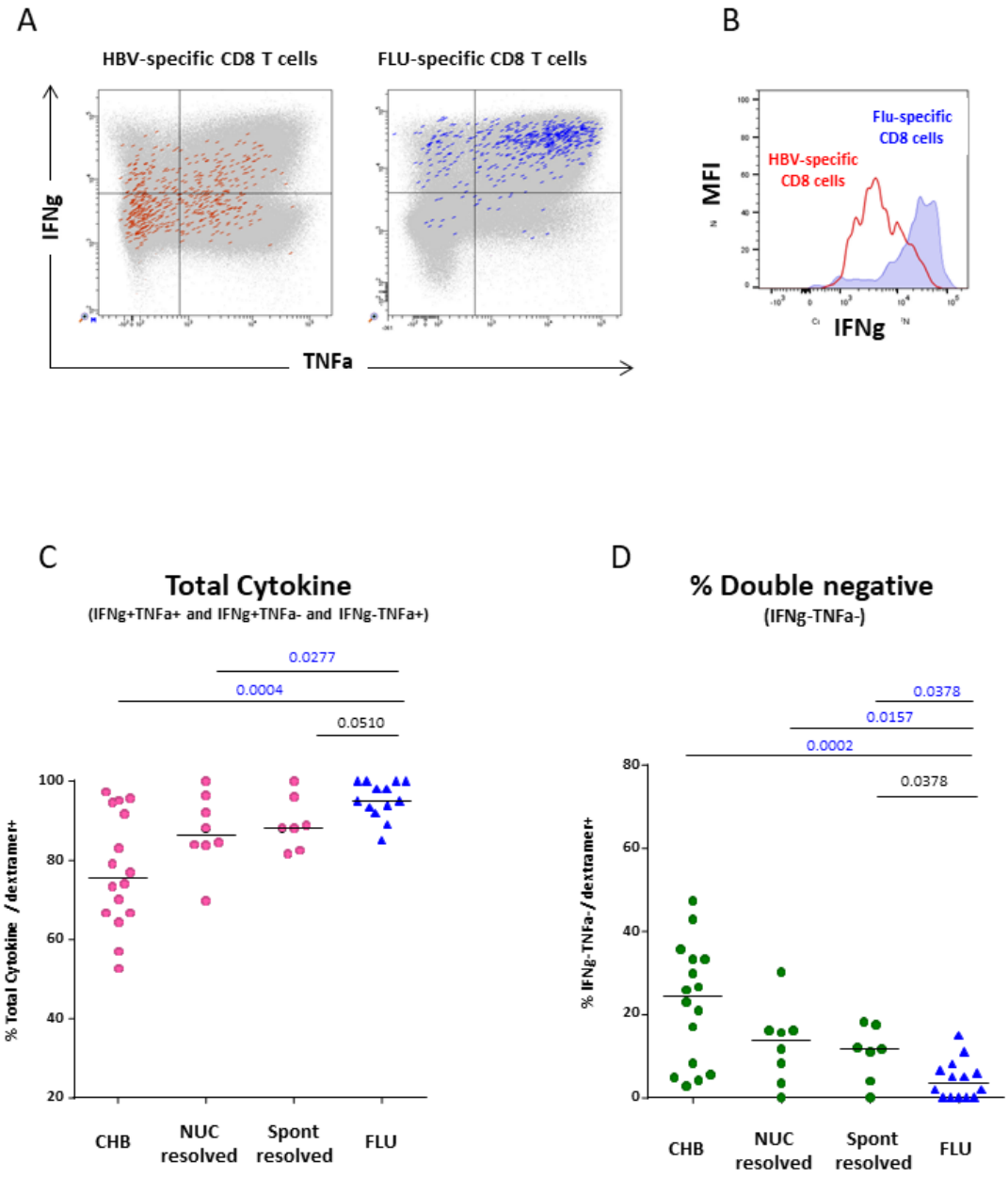

Figure 13. *Ex Vivo* HBV-specific T Cell Functional Analysis. Intracellular cytokine staining by 4 hours PBMCs stimulation with PMA + Ionomycin analyzed on dextramer positive HBV-specific CD8 T cells. A) Representative plots showing the percentage of HBV-specific (on the left) and Flu-specific CD8 T cells (on the right). B) Same results represented as Mean Fluorescence Intensity (MFI). C) The total percentage of HBV-specific (pink dot) and FLU-specific (blue dot) CD8 T cells able to produce cytokines are illustrated in naive viremic CHB patients, NUC resolved patients, spontaneous controllers and flu-pecific CD8 cells derived from each individual patient and healthy subject. Each symbol represents the total frequency of IFN-γ and TNF-α positive HBV-specific CD8 cells calculated in each patient. D) Percentage of double negative (IFNg-TNFa-) HBV-specific (green dot) and FLU-specific (blue dot) CD8 T cells in the same patient groups. Statistically significant differences between different patient categories detected by the Mann-Whitney test are indicated.

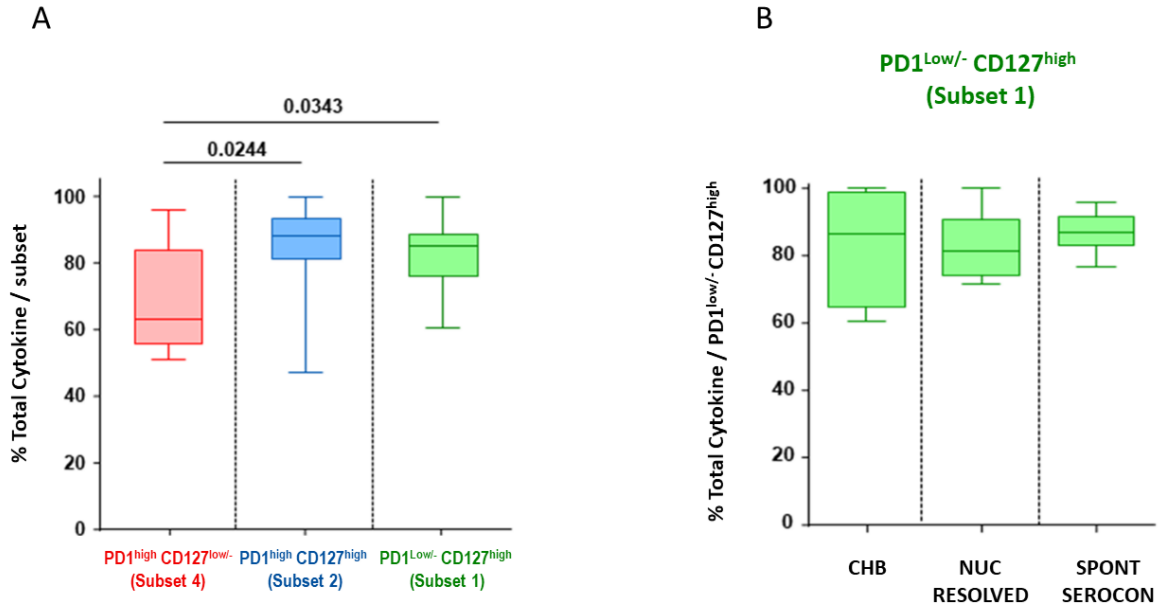

Figure 14. *Ex Vivo* HBV-specific T Cell functional characterization of the different CD8 T cell subsets. A) The median percentage of HBV-specific CD8 T cells able to produce cytokines are illustrated for the three CD8 T cell subsets: PD1^{high} CD127$^{low/-}$ cells (subset 4, red bar), PD1$^{low/-}$ CD127^{high} (subset 1, green bar), PD1^{high} CD127^{high} (subset 2, blue bar). The panel illustrates data derived from all analyzed patient populations. Statistically significant differences between different subsets were assessed by the Mann-Whitney test. B) Median percentage of PD1$^{low/-}$ CD127^{high} HBV-specific CD8 T cells able to produce cytokines (subset 1, green bar) in the different patient categories. No statistical difference was found by the Mann-Whitney test.

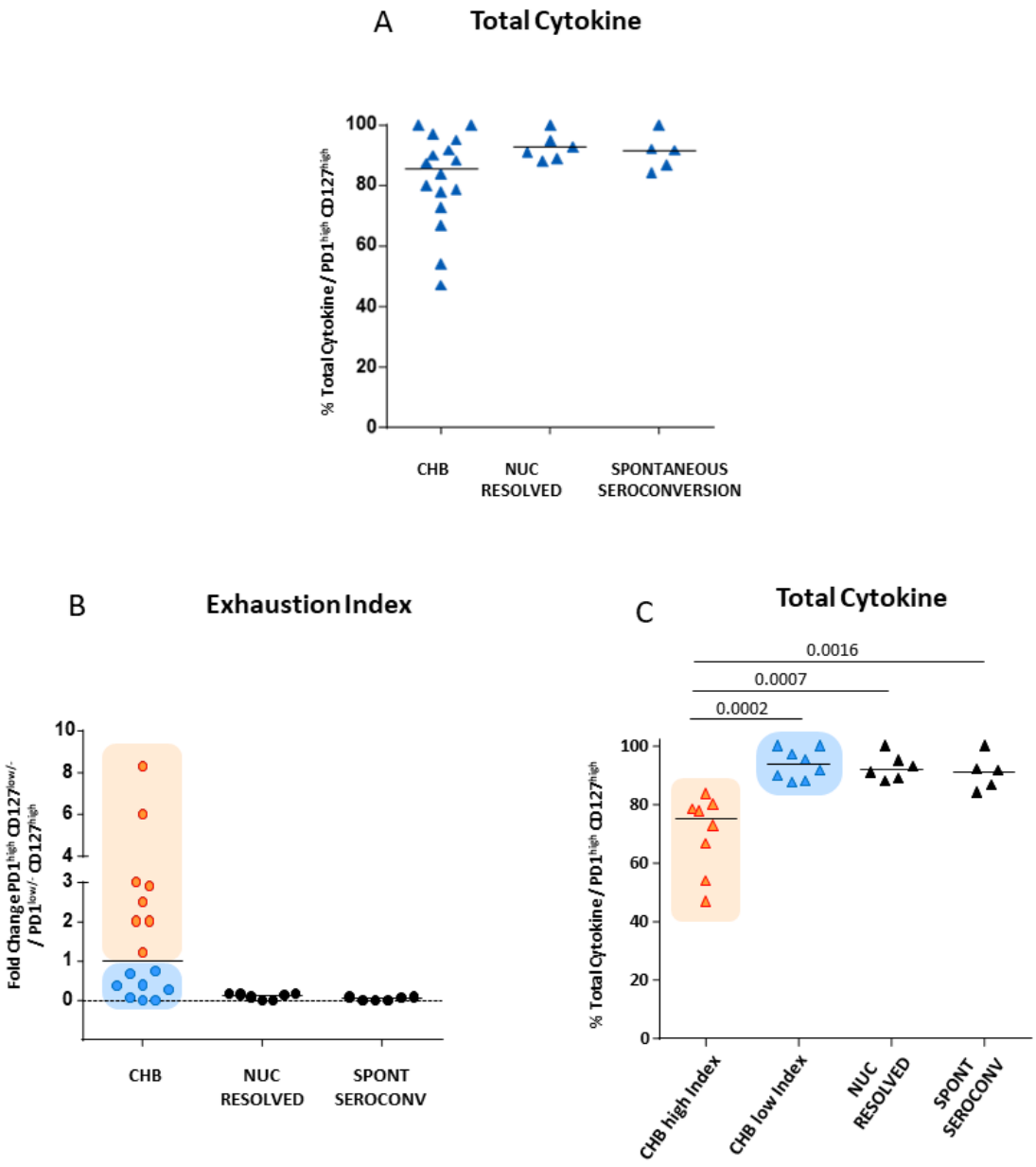

Figure 15. Correlation between cytokine production by PD1^{high} CD127^{high} HBV-specific CD8 T cells and exhaustion index values in different patient categories. A) Total percentage of PD1^{high} CD127^{high} HBV-specific CD8 T cells able to produce cytokines in naive viremic CHB, NUC resolved patients and spontaneous controllers. Each symbol represents the total frequency of IFN-γ and TNF-α positive PD1^{high} CD127^{high} HBV-specific CD8 cells calculated in each patient. B) *Exhaustion Index* (EI) values are illustrated for naive viremic CHB (orange and blue dots), NUC resolved patients and spontaneous controllers (black dots). Each dot represents the EI calculated for each individual patient. CHB patients were separated into two groups based on their EI levels: high EI (>1) in orange, low EI (<1) in blue. C) Total percentage of HBV-specific CD8 T cells able to produce cytokines in the same patients illustrated in B. Each symbol represents the total frequency of IFN-γ and TNF-α positive HBV-specific CD8 cells calculated for each patient. Patients represented in blu and orange are the same illustrated with the same color in panel B. Statistically significant differences between different patient categories were assessed by the Mann-Whitney test.

Combined analysis of functional/phenotypic profiling of HBV-specific CD8 T cells.

Based on this correlation between phenotypidc and functional profiling we then tried to confirm and refine the predictive value of the *Exhaustion Index,* by calculating in each patient the ratio between the percentage of total cytokine production by $PD1^{high}$ $CD127^{high}$ HBV-specific CD8 T cells and the percentage of double negative cells among this subset (figure 16 A). The number generated by this calculation is defined as *Functional Index* and higher values express better functionality, as opposed to the *Exhaustion Index* which instead associates better functionality with low SI values.

As predictable, a higher *Functional Index*, indicative of better functionality, was expressed by the same CHB patients with lower exhaustion index by phenotypic analysis (figure 6). As illustrated in figure 16 B, a statistically significant inverse correlation between the values of the two indexes (the functionality and the exhaustion ratio) was observed, showing a nice segregation of the less *exhausted* naive CHB patients (red dots) within the group of resolved subjects (blue dots). Interestingly, the better T cell responsiveness of the less *exhausted* chronic patients was not associated with lower serum antigen levels because variable HBsAg concentrations were detected in these patients.

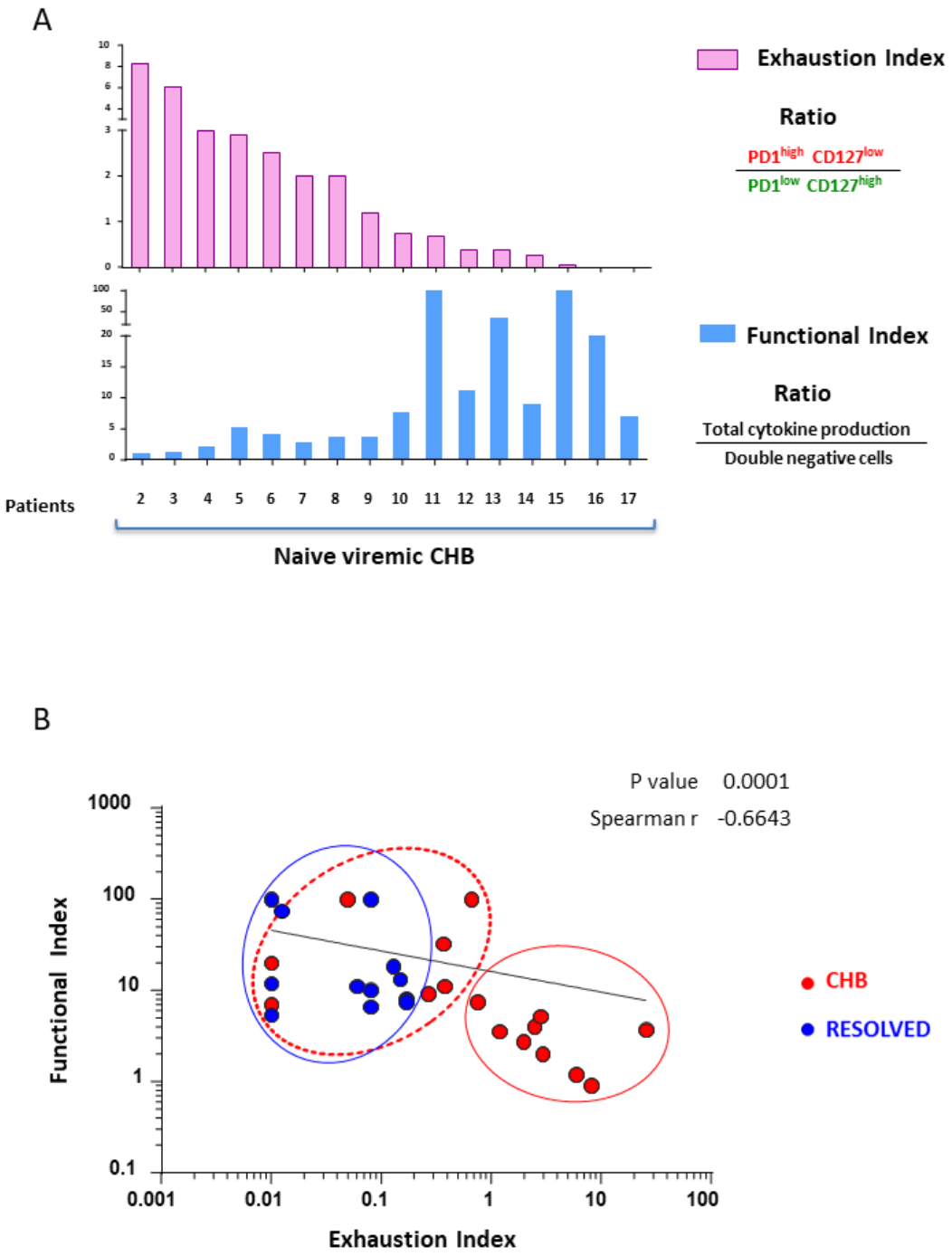

Figure 16. Independent prediction of CD8 T cell exhaustion/functionality by phenotypic and functional analysis. A) Representation of *Exhaustion Index* (defined by the ratio between the percentage of PD1^{high} CD127^{low} and PD1$^{low/-}$ CD127^{high} cells; pink bars) and *Functional Index* (defined by the ratio between total cytokine production by PD1^{high} CD127^{high} HBV-specific CD8 cells and the percentage of double negative cells among this subset; blue bars) in chronic hepatitis B patients. B) Correlation between functional Index (calculated in PD1^{high} CD127^{high} HBV-specific CD8 T cells) and Exhaustion Index. Red dots represent chronic viremic naive subjects while blue dots identify HBV resolver patients (anti-HBs+ NUC resolved patients and spontaneous seroconversions). Statistically significant values were assessed by the Spearman's correlation test.

Additional exhaustion and differentiation markers increase the predictive power of the PD-1/CD127 based algorithm.

In order to further improve the predictive capacity of the PD-1/CD127 based algorithm, additional exhaustion and differentiation/memory phenotypic parameters were simultaneously stained in HBV-specific T cell subsets, including the transcriptor factor TCF1, needed for differentiation and establishment of memory CD8 T cells, the anti-apoptotic Bcl-2 molecule, the ectonucleotidase CD39, and the exhaustion-related molecule TOX, described in association with the progression of T-cell dysfunction and with the maintenance of exhausted T-cells during chronic infection.

In accordance with previous reports derived from different settings of chronic virus infection in humans and mice [70], CD39 expression by HBV-specific CD8 T cells were detected only in chronically infected patients at variable individual levels with a direct correlation with the frequency of the most exhausted $PD1^{high}$ $CD127^{low}$ CD8 T cell subset (figure 17). Notably, a group of CHB patients showed lower CD39+/$PD1^{high}$ $CD127^{low}$ co-expression levels similar to those observed in NUC and spontaneous resolvers.

TCF-1, which is known to have a key role in the long-term maintenance of T cell responses in chronic infections [65] was analyzed in the different CD127/PD-1 HBV specific CD8 T cell subsets in naïve viremic CHB patients. Higher levels of TCF-1 were detected among the $PD1^{low/-}$ $CD127^{high}$ CD8 T cell subset (medium fluorescence intensity - MdFI - 1070) compared to $PD1^{high}$ $CD127^{low/-}$ (MdFI 705) and $PD1^{high}$ $CD127^{high}$ (MdFI 864) T cell subpopulations. Of note, TCF1 expression was significantly reduced in the *exhausted* $PD1^{high}$ $CD127^{low/-}$ cell subset in comparison with the less impaired $PD1^{low/-}$ $CD127^{high}$ T cell subpopulation. Moreover, TCF-1 expression in NUC-resolved and spontaneous controllers was predominantly distributed within the $PD1^{low/-}$ $CD127^{high}$ and $PD1^{high}$ $CD127^{high}$ T cell

subsets, suggesting memory-like characteristics of double-positive TCF1+/CD127+ cells highly represented in these patient categories (figure 18 A).

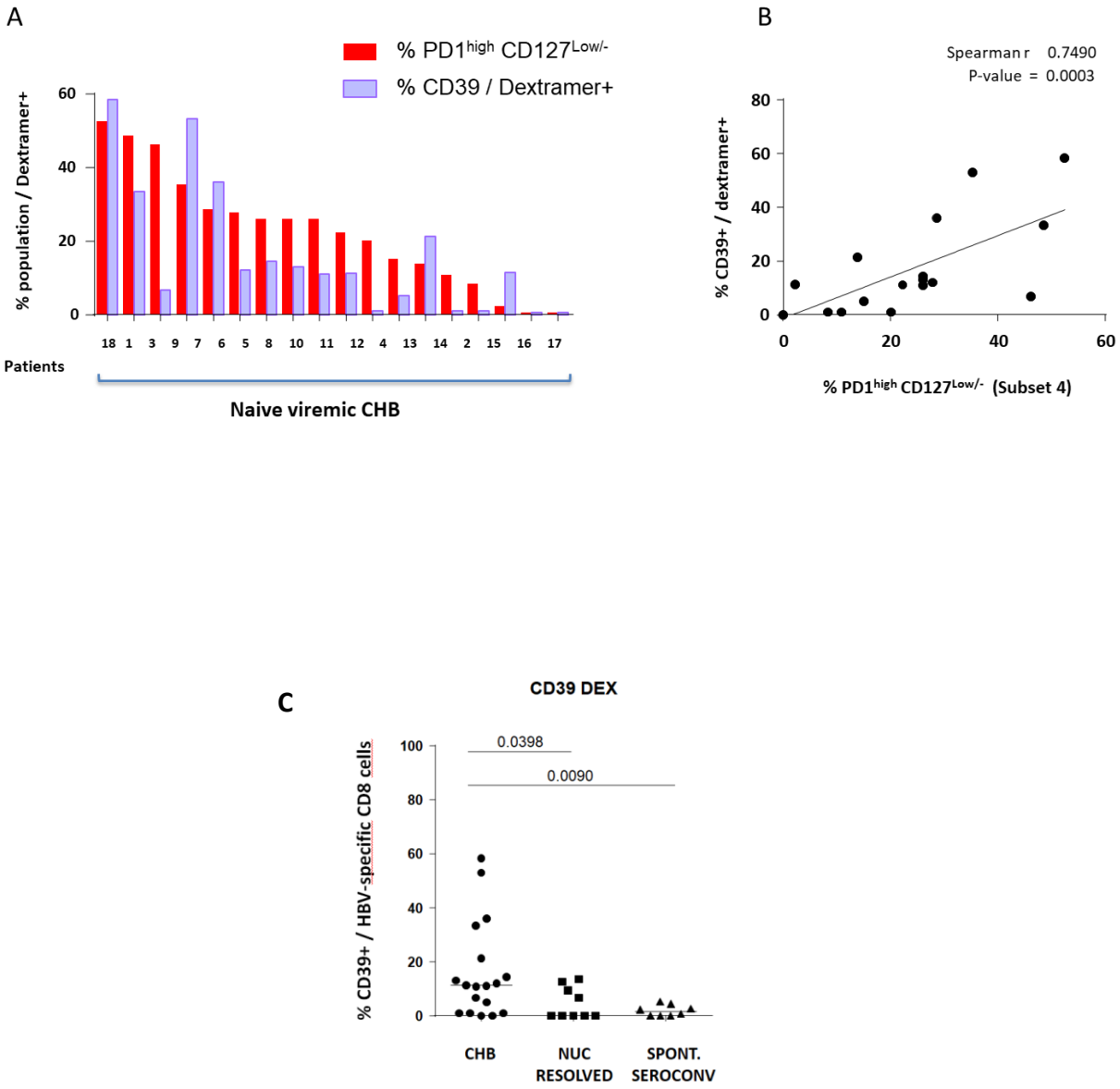

Figure 17. CD39 expression on HBV-specific CD8 T cells from viremic CHB patients. A) CD39 (red bars) and PD1^{high} CD127$^{low/-}$ (red bars) expression on HBV-specific dextramer+ CD8 T cells from naive viremic CHB patients. B) Direct significant correlation between CD39 expression levels and frequency of PD1^{high} CD127^{low} CD8 T cells among HBV-specific T cells. Each dot represents individual CHB patients. Statistics was performed by the Spearman's correlation test. C) Frequency of CD39 positive HBV-specific CD8 T cells in the different patient categories. Each dot represents individual subjects; the statistical comparison between different groups was done by the Mann-Whitney test .

The HMG-box transcription factor (TOX) is another key molecule involved in promoting T cell exhaustion [75,76] and TOX induction by strong and persistent antigen T-cell receptor stimulation has been reported during mice LCMV and human HCV infections [76]. TOX expression was significantly increased in the more *exhausted* $PD1^{high}$ $CD127^{low/-}$ cell subset in comparison with the less exhausted $PD1^{low/-}$ $CD127^{high}$ T cell subpopulation in chronically infected patients. Conversely, TOX expression was either poorly or totally not expressed in all different T cell subsets derived from NUC and spontaneous resolvers (figure 18 B).

Finally, the survival capacity of the different HBV-specific CD8 T cell subsets was analyzed by looking at the expression of the anti-apoptotic marker Bcl-2. In keeping with TCF1 expression, $PD1^{high}$ $CD127^{low/-}$ CD8 T cells exhibited the lowest levels of Bcl-2 (MdFI 3630) compared to both $PD1^{low/-}$ $CD127^{high}$ (MdFI 8055) and $PD1^{high}$ $CD127^{high}$ (MdFI 7546) T cell subpopulations (figure 8 C). These data are consistent with recent results derived from chronic HCV patients showing lower expression of Bcl-2 in the terminallydifferentiated $PD1^{high}$ $CD127^{low/-}$ CD8 T cell subset [65].

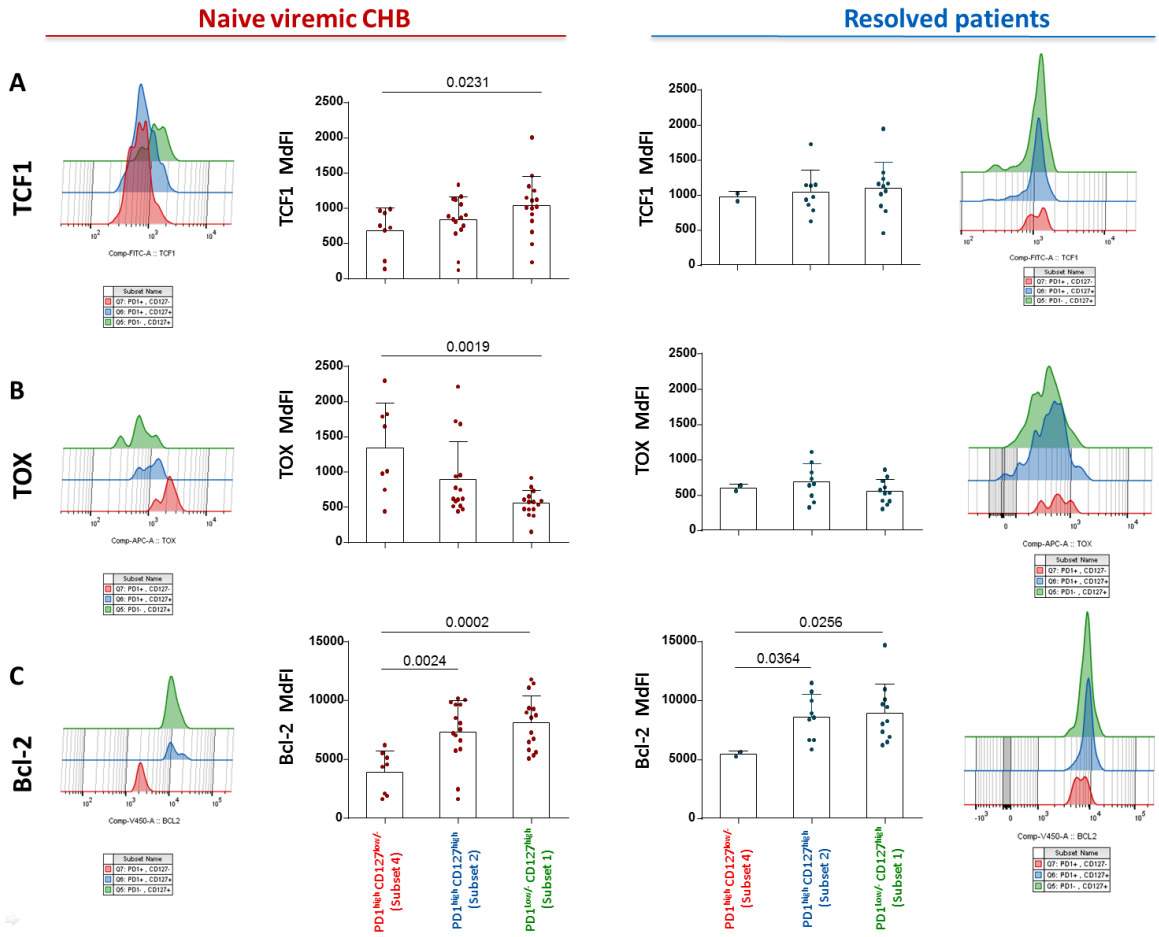

Figure 18. Expression of TCF1, TOX and Bcl-2 in different HBV-specific CD127/PD-1 CD8 T cell subsets from viremic CHB and resolved patients. Expression of TCF1 (A), TOX (B) and Bcl-2 (C) represented as Median Fluorescence Intensity (MdFI) in different HBV-specific CD127/PD-1 CD8 T cell subsets in naive viremic CHB (red dots) and NUC resolved or spontaneous controllers (blue dots). A representative histogram plot is represented for each marker ($PD1^{high}$ $CD127^{low/-}$ in red, $PD1^{high}$ $CD127^{high}$ in blue and $PD1^{low/-}$ $CD127^{high}$ in green). Statistically significant differences between HBV-specific CD8 T cell subsets were assessed by the Mann-Whitney test.

DISCUSSION

Exhausted HBV-specific CD8 T cells are a composite dysfunctional population [44,48] which requires to be better characterized in order to unravelits phenotypic and functional heterogeneity in chronic HBV patients and to understand whether its composition at individual patient level can be helpful to predict response to immune modulators.

For this purpose, we performed an in-depth phenotypic and functional analysis of HBV-specific CD8 T cells directly ex vivo in a well-characterized population of eAg-negative chronic naive patients with high viral load and fluctuating ALT levels. We also extended the phenotypic and functional profiling to NUC-treated patients with complete control of infection and to spontaneous controllers following long-time chronic carriage of the virus, as reference patient groups able to develop anti-HBs antibodies and to obtain viral control . Our study of chronic HBV patients either untreated and viremic or resolved after long-term infection adds to the existing knowledge a series of novel and important findings. First, by using a dextramer-based approach, $Core_{18-27}$ specific CD8 T cells were the only T cell population detectable in the majority of chronic viremic HBV-infected patients, while Pol_{455} and Env_{335}-specific CD8+ T cells were selectively found in HBV resolver patients, likely suggesting an higher degree of exhaustion by CD8 T cells targeting polymerase and evelope proteins. This finding is in agreement with previous reports indicating that polymerase-specific CD8 T cells exhibit a more severely impaired function in CHB patients with low levels of viral load [78].

CD8 T cells specific for Core 18-27 were characterized *ex vivo* by simultaneous staining with different exhaustion (PD1, CD39, TOX) and memory (CD127, BCL2) markers in combination with specific transcriptional factors (TCF1). Importantly, our study indicates

that a subset of PD1^{high} and CD127$^{low/-}$ HBV-specific CD8 T cells was selectively detectable in untreated viremic patients, while PD1low/- and CD127^{high} CD8 cells were preferentially represented in NUC resolved patients and spontaneous controllers. This more protective profile was also detected in a limited proportion of chronic viremic patients who are likely more prone to respond to immune modulation. Although spontaneous or treatment-induced control of infection allowed HBV-specific CD8 cells to acquire a less exhausted phenotype, their profile however did not become identical to that of flu-specific CD8 cells, almost totally represented by PD1$^{low/-}$ CD127^{high} cells. This is consistent with data generated in chronic HCV infection where TCF1+CD127+PD-1+ memory-like CD8 T cells were maintained after therapy-mediated HCV clearance, retaining high levels of PD-1 and Eomes associated with reduced cytokine production in comparison with conventional memory HCV-specific T cells generated in patients who spontaneously resolved HCV infection [65]. Intriguingly, also in LCMV chronically infected mice virus-specific TCF1+ T cells with high proliferative capacity showed a distinctive transcriptome profiling resembling features of both memory and exhausted T cells [60,82].

Finally, a subset of PD1^{high} CD127^{high} HBV-specific CD8 cells was present in all groups of patients, irrespective of the clinical condition and level of virus control. Thus, our phenotypic analysis reveals that exhausted HBV-specific T cells in chronic hepatitis B patients exhibit a heterogeneous molecular profiling consisting of three different T cell subsets according to the expression of the inhibitory receptor PD-1 and the T cell memory-related marker CD127. Based on available results in other infection models, the prevalence of the PD1$^{low/-}$ CD127^{high} over PD1^{high} CD127^{low} HBV-specific CD8 T cell subset in individual patients should give indication of better protection, allowing to select the patients with different degrees of exhaustion. To confirm the potential predictive value of

this phenotypic parameters, we characterized ex vivo the functional features of HBV-specific CD8 T cell sub-populations and correlated them with their phenotypic profile. As expected, we observed lower cytokine levels produced by HBV-specific CD8 T cells from chronic patients as compared to resolvers and to flu-specific CD8 cells. In line with our prediction, we also found functional differences between distinct T cell subsets in chronic patients because the $PD1^{high}CD127^{low/-}$ T cell subsets showed a significantly lower cytokine production compared to $PD1^{low/-}CD127^{high}$ HBV-specific CD8 T cells.

Comparison of functional and phenotypic profiling revealed that a higher cytokine production, indicative of better functionality, was expressed by the same CHB patients with a less exhausted phenotype. Importantly, the *Exhaustion Index* allowed to identify two groups of CHB patients, one of them with features more similar to resolved patients.

Next, simultaneous analysis of additional exhaustion and memory/differentiation markers allowed to further increase the predictive power of the PD-1/CD127 based algorithm. The $PD\text{-}1^{high}CD127^{low/-}$ HBV-specific T cell subset clearly correlated with the levels of the ecto-nuclease CD39, responsible for the generation of immune suppressive adenosine. These results are in agreement with previous studies perfomed in LCMV-infected mice and in chronically HCV- and HIV-infected patients reporting that CD39 expression identifies terminally exhausted CD8 T cells [70]. Moreover, since recent findings demonstrated a key role for the HMG-box transcription factor (TOX) in promoting and maintaining T cell exhaustion [75,76], in addition to PD-1 and CD39 we also quantified TOX expression in the different HBV-specific T cell subsets . Noteworthy, TOX level was significantly increased in the exhausted $PD1^{high}$ $CD127^{low/-}$ cell subset in comparison with the less impaired $PD1^{low/-}$ $CD127^{high}$ T cell subpopulation among chronically infected patients, allowing to further improve the generation of prediction algorithms of immunotherapy response.

Furthermore, the proliferative capacity and the T cell survival were determined by using the memory-related transcription factor TCF1 and the anti-apoptotic marker Bcl-2, respectively. Of note, we observed that both TCF1 and Bcl-2 were highly expressed by the less impaired HBV-specific CD127^{high} T cell subsets either in chronic or spontaneously resolved patients. These results are in line with previous report in HCV infection showing that the memory-like CD127+PD1+ CD8 T cells strongly expressed TCF1 and Bcl-2 [65]. Interestingly, the same authors reported a predominance of a memory-like phenotype by both HBV core- and polymerase-specific CD8 T cells in chronic HBV patients with low viral load, showing a reduced expansion capacity linked to a dysregulated TCF1/Bcl-2 expression of polymerase-specific CD8 T cell population [78,83].

Overall these results suggest a divergent T-cell differentiation program which likely underpins the induction of T cell exhaustion during chronic infections and which cannot be rewired despite viral clearance [56]. Moreover, in the murine LCMV model of chronic infection a minimal transcriptome remodeling was observed in exhausted CD8 T cells upon PD-L1 blockade; these cells maintain a peculiar epigenetic profile that could represent a critical issue for the definition of immunomodulatory strategies [57].

In conclusion, by simultaneous staining with different exhaustion and memory markers in combination with specific transcriptional factors, we were able to identify different HBV-specific CD8 T cell subsets, characterized by different phenotypic profiles and endowed with different levels of exhaustion which can co-exist in individual infected hosts. The relative predominance of more or less exhausted T cell subsets seems to dictate the level of functional efficiency of the overall anti-viral CD8 T cell population. The patients who harbor a less inhibited virus-specific immune system with predominance of less exhausted CD8 T cell subsets may identify the patient cohort with more likelihood of

response to therapeutic immune modulatory interventions. A deeper analysis of metabolic and transcriptional features of individual HBV-specific CD8 T cell subsets are needed to uncover novel T cell exhaustion mechanisms, to identify optimal molecular targets for functional T cell restoration strategies and to generate prediction algorithms of response to immune modulatory interventions based on CD8 cell phenotypic profiling.

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