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CD8 T cell phenotypic and functional characterization to identify immunological predictors of response to immune modulatory treatments in chronic HBV infection

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ABSTRACT

Objective: Exhausted hepatitis B virus (HBV)-specific CD8 T cells in chronic HBV infection are broadly heterogeneous. Characterisation of their functional impairment may allow to distinguish patients with different capacity to control infection and reconstitute antiviral function.

Design: HBV dextramer+CD8 T cells were analysed ex vivo for coexpression of checkpoint/differentiation markers, transcription factors and cytokines in 35 patients with HLA-A2+chronic hepatitis B (CHB) and in 29 control HBsAg negative CHB patients who seroconverted after NUC treatment or spontaneously. Cytokine production was also evaluated in HBV peptide-stimulated T cell cultures, in the presence or absence of antioxidant, polyphenolic, PD-1/PD-L1 inhibitor and TLR-8 agonist compounds and the effect on HBV-specific responses was further validated on additional 24 HLA-A2 negative CHB patients.

Results: Severely exhausted HBV-specific CD8 T cell subsets with high expression of inhibitory receptors, such as PD-1, TOX and CD39, were detected only in a subgroup of chronic viraemic patients. Conversely, a large predominance of functionally more efficient HBV-specific CD8 T cell subsets with lower expression of coinhibitory molecules and better response to in vitro immune modulation, typically detected after resolution of infection, was also observed in a proportion of chronic viraemic HBV patients. Importantly, the same subset of patients who responded more efficiently to in vitro immune modulation identified by HBV-specific CD8 T cell analysis were also identified by staining total CD8 T cells with PD-1, TOX, CD127 and Bcl-2.

Conclusions: The possibility to distinguish patient cohorts with different capacity to respond to immune modulatory compounds in vitro by a simple analysis of the phenotypic CD8 T cell exhaustion profile deserves evaluation of its clinical applicability.

The information presented in this thesis has been previously published in the article: Rossi M, Vecchi A, **Tiezzi C**, et al. Gut 2023;72:2123–2137[67]

INTRODUCTION

1. Hepatitis B

1.1 Viral genome, replication, and genotypes.

The hepatitis B virus (HBV) is a member of the Hepadnaviridae family and its viral genome is characterized by a partially double-stranded circular DNA genome, with a size of 3.2 kb. By electron microscopy, the HBV virion appears as a small spherical particle, approximately 42-47 nm as diameter (Dane particle), consisting of a lipoprotein coat (envelope) and a nucleocapsid core with icosahedral symmetry, which encloses the viral genome and the endogenous DNA polymerase (Figure 1)[1].

The nucleotide sequence analysis of the HBV genome has revealed the presence of four partially overlapping Open Reading Frames (ORFs) that encode the structural and non-structural proteins of the virion: P, S, C, and X[2].

The P (Polymerase) ORF corresponds to 70% of the viral genome and encodes the virus-specific DNA polymerase. This region is functionally divided into three domains:

1. the terminal protein (TP) domain, involved in binding of the recognition sequence at the 5' end of pre-genomic RNA, ensuring the initiation of the synthesis of the negative strand of HBV DNA.
2. the reverse transcriptase (RT) domain, encoding the RT enzyme, which has DNA and RNA-dependent polymerase activity.
3. the ribonuclease H (RNAsiH) domain, involved in the degradation of pre-genomic RNA and facilitating viral replication[3].

The S (Surface) ORF encodes envelope proteins (HBsAg). Specifically, this region can be structurally and functionally divided into pre-S1, pre-S2, and S regions. This allows the synthesis of three different viral surface antigens, based on the starting point of gene translation (Figure 1): the S gene encodes SHBsAg (small protein), the pre-S2 + S genes encode MHBsAg (medium protein), and the pre-S1 + pre-S2 + S genes encode LHBsAg (large protein).

The C (core) ORF includes both core and pre-core regions, and depending on the initiation of translation from C or pre-C region, it encodes either the core protein (HBcAg) or the E antigen

(HBeAg), a non-structural protein that is secreted into the serum of infected patients and serves as a marker of active viral replication.

Finally, the X ORF encodes the X protein, which has multiple functions, including signal transduction, transcriptional activation, DNA replication, and inhibition of protein degradation. The mechanism of this activity and the biological function of HBxAg in the viral replication cycle remain largely unknown; however, it is known that this protein plays a role in the infection itself and may contribute to the oncogenic potential of HBV[4–6].

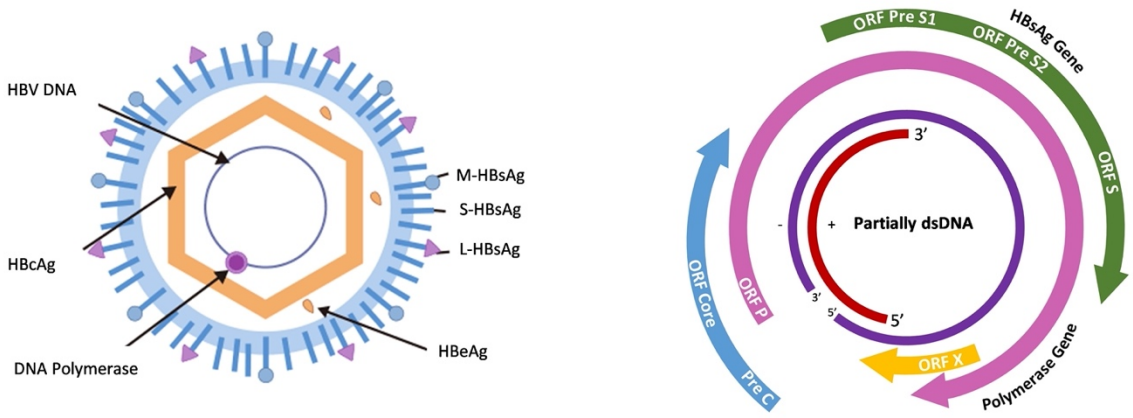

Figure 1. Viral particle and genome structure of HBV. On the left, the morphology of the viral particle is shown. On the right, the structure of the HBV genome is displayed; in the inner part, the partially double-stranded DNA is depicted, and externally, the four ORFs (Open Reading Frames) coding for the different viral proteins are represented with arrows.

HBV, despite being a DNA virus, has a replication cycle similar to that of retroviruses, as it involves the reverse transcription of an RNA intermediate, called pre-genomic RNA. Specifically, the replication cycle of HBV can be divided into three phases (Figure 2)[7]: in the first phase, the virions, containing the entire viral genome in a relaxed circular form (RC-DNA), penetrate the host cell; in the second phase, using cellular enzymes, the RC-DNA is transferred to the cell nucleus and converted into covalently closed circular DNA (cccDNA), similar to a plasmid; finally, in the third phase, the actual infection begins due to the presence of cccDNA, which is used as a template for the transcription of various pre-genomic and sub-genomic RNAs by cellular RNA polymerase II. The mRNAs and pre-genomic RNAs (pgRNA) are then transferred to the cytoplasm where they will be used for the synthesis of structural and non-structural proteins necessary for the assembly of new virions.

Indeed, pre-genomic RNAs are bi-functional as they represent both the messengers for the production of capsid and polymerase proteins, and the templates for the synthesis of progeny viral DNA, thanks to reverse transcriptase.

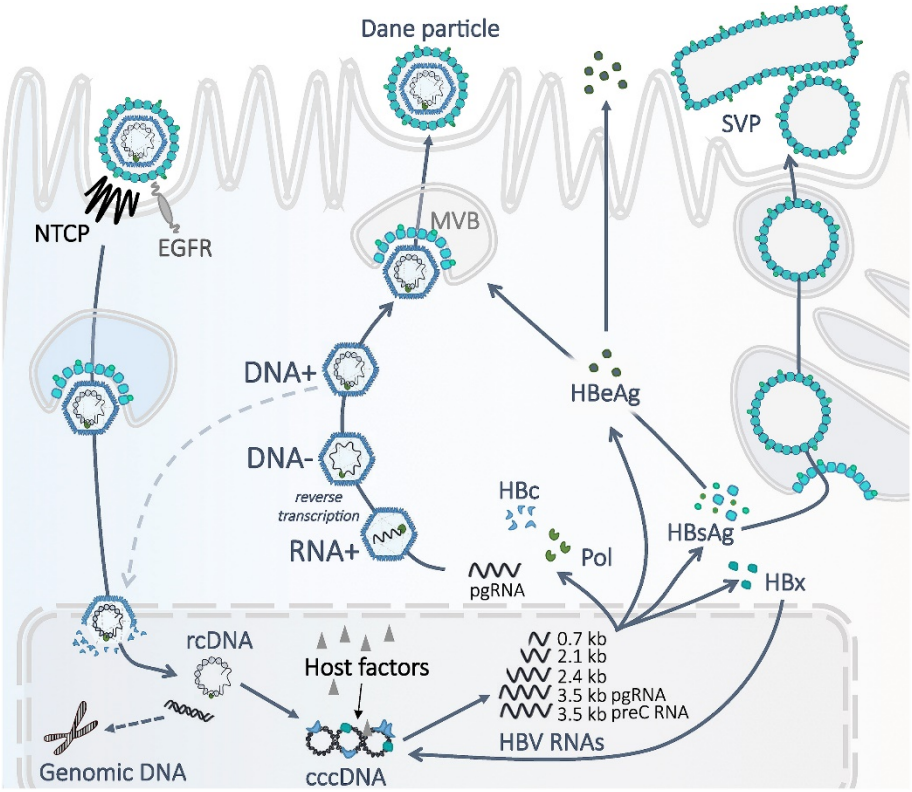

Figure 2. Schematic overview of the HBV life cycle. Source: Senko Tsukuda and Koichi Watashi. Hepatitis B virus biology and life cycle. Antiviral Res. 2020 Oct:182:104925. doi: 10.1016/j.antiviral.2020.104925.

Genomic DNA sequencing has also revealed the presence of numerous different viral genotypes, with a distribution that varies according to different geographical areas[8,9]. To date, 10 viral genotypes have been identified, classified from A to J, and numerous sub-genotypes with nucleotide sequence differences greater than 8% for genotypes and between 4% and 8% for sub-genotypes, which can differently affect disease progression, clinical course, response to antiviral treatment, and prognosis (Figure 3).

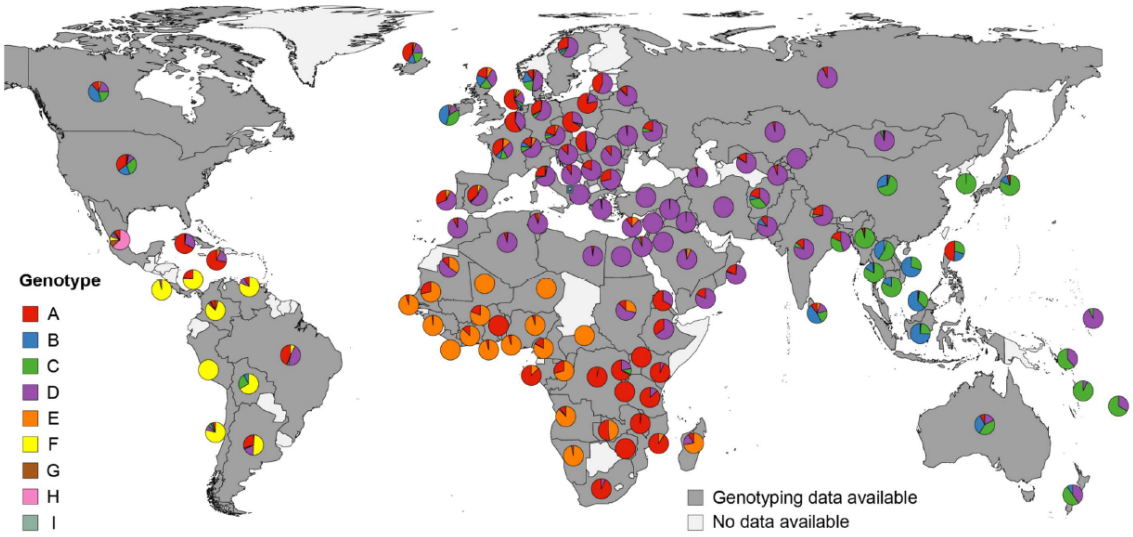

Figure 3. Distribution of HBV genotypes by country. Pie charts indicate proportional HBV genotype distributions in the respective countries. Source: Velkov, S.; Ott, J.J.; Protzer, U.; Michler, T. The Global Hepatitis B Virus Genotype Distribution Approximated from Available Genotyping Data. Genes 2018, 9, 495. doi:10.3390/genes9100495

Genotype A is widespread in sub-Saharan Africa, Northern Europe, and Western Africa; genotypes B and C are common in Asia; genotype D is prevalent in Europe, Africa, the Mediterranean basin, and India; genotype G is endemic in France, Germany, and the USA; genotype H in Central and South America; and finally, genotypes I and J are found, respectively, in Vietnam[10] and Laos, and in Japan in the Ryukyu Islands[8].

1.2 Epidemiology and public health burden

Hepatitis B virus (HBV) infection is a significant global health problem. It is one of the primary causes of chronic liver disease, cirrhosis, and hepatocellular carcinoma (HCC), leading to over 600,000 deaths annually[11]. According to the World Health Organization (WHO), over 2 billion people worldwide have been infected with HBV, and more than 350 million of these are carriers of HBsAg[12]. The prevalence and transmission modes of HBV infection vary widely across the world, depending on different geographical areas (Figure 4)[13].

Transmission most commonly occurs from mother to child at birth (perinatal transmission) or through exposure to infected blood and bodily fluids (such as semen and vaginal secretions), although the presence of the virus has also been detected in saliva, tears, breast milk, sweat, and urine.

In countries where chronic HBV infection affects more than 8% of the population, most individuals were infected at birth or during early childhood when the risk of progression to chronicity is high. In industrialized countries, higher prevalence is seen among immigrants and individuals with high-risk behaviors, such as intravenous drug use or promiscuous sexual activity. Italy falls into the low endemic region, with a prevalence of chronic infections around 1%. This is largely due to the development of the HBV vaccination program, which began in 1991 and is mandatory for newborns, significantly reducing the rate of new infections[14].

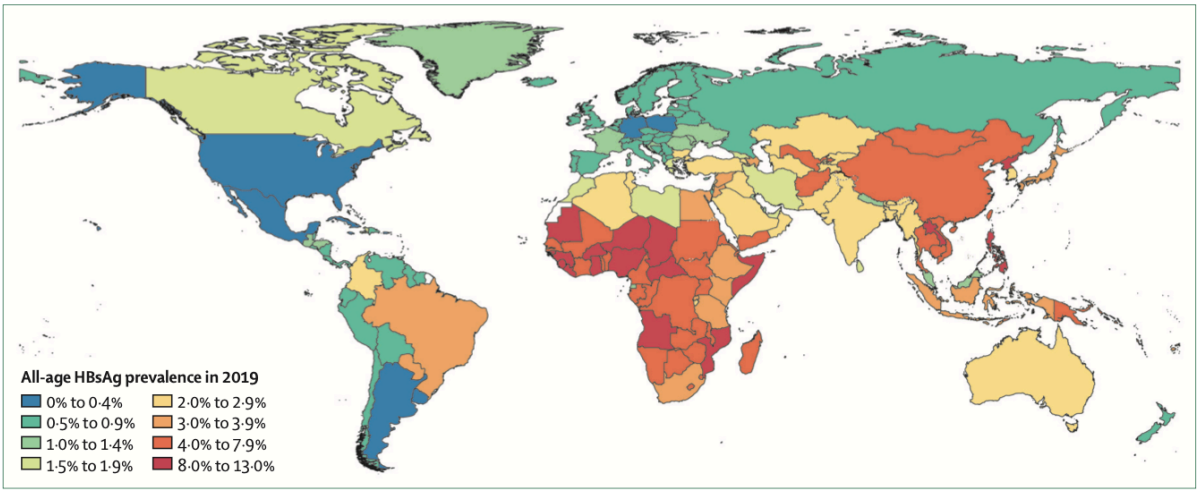

Figure 4. Map of the global prevalence of HBV infection. Source: Jeng WJ, Papatheodoridis GV, Lok ASF. Hepatitis B. Lancet. 2023 Mar 25;401(10381):1039-1052. doi: 10.1016/S0140-6736(22)01468-4. Epub 2023 Feb 9. PMID: 36774930

1.3 Natural History of Infection

Chronic HBV infection is a dynamic process that reflects the interaction between viral replication and the host's immune response. The natural history of chronic HBV infection has been schematically divided into five different phases, considering the presence of HBeAg, HBV-DNA levels, transaminase (ALT) levels, and the presence or absence of liver inflammation (Figure 5)[13].

Specifically, during acute HBV infection, symptoms and outcome depend on age of infection acquisition, the level of viral replication, and the individual's immune status. The age at the time of virus exposure appears to be the most important determinant for the chronicity of the infection[15]. In fact, while infection acquired in adulthood is predominantly asymptomatic and results in complete recovery with viral clearance in 90-95% of cases, exposure to the virus during childhood is associated with much higher rates of chronicity. The percentage of chronicity increases the earlier the infection occurs: it is around 90-95% in individuals who contracted the virus perinatally, decreases to 25-30% in patients infected within the first five years of life, and drops to a 5-10% probability of chronicity in adults, where self-limiting infection predominates[15].

The new nomenclature is based on describing the two main characteristics of chronicity, namely the presence of infection or hepatitis[16]. However, despite this nomenclature, it is not always possible to immediately classify an individual into one of the phases.

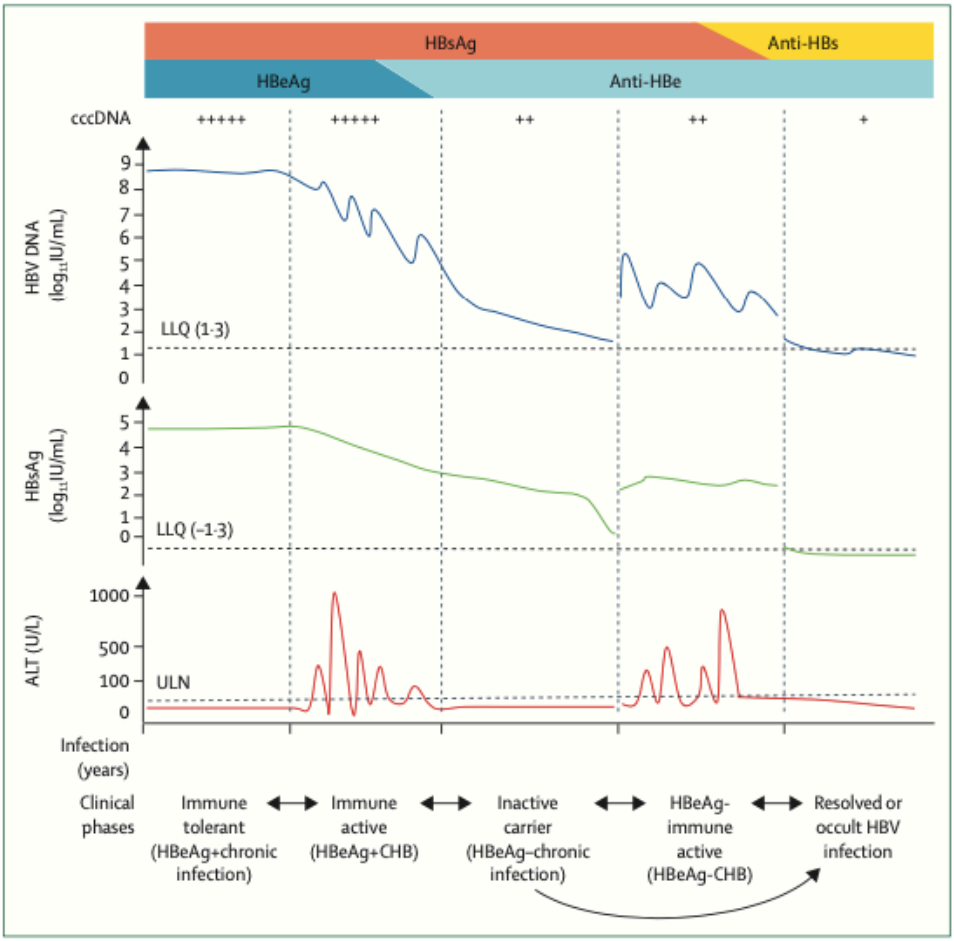

Figure 5. Natural history of hepatitis B virus infection. Source: Jeng WJ, Papatheodoridis GV, Lok ASF. Hepatitis B. Lancet. 2023 Mar 25;401(10381):1039-1052. doi: 10.1016/S0140-6736(22)01468-4. Epub 2023 Feb 9. PMID: 36774930

The phases of chronic HBV infection are not necessarily sequential (Table 1):

Phase 1: Previously called the "immune tolerance phase," this phase is characterized by high viral replication with elevated HBV-DNA levels in the serum (>10^7 IU/mL), HBeAg positivity, normal serum transaminase (ALT) levels, and minimal liver inflammation. This phase is more common in individuals infected perinatally and can persist for 20-40 years, whereas it is short or absent in infections acquired later in life. Additionally, patients are highly contagious due to the high levels of viral DNA[17,18].

Phase 2: Previously called the "immune clearance phase," this phase is characterized by high HBV-DNA levels, HBeAg positivity, elevated ALT levels, and moderate to severe necroinflammation of the liver. This phase can occur several years after the first phase and is more frequently and/or rapidly reached in individuals infected during adulthood. In most

patients, this phase ends with the loss of HBeAg positivity through spontaneous seroconversion and the development of anti-HBe antibodies. After seroconversion, about 70% of individuals will enter in the inactive carrier phase, about 20% will revert to an HBeAg-positive state, often accompanied by reactivation of hepatitis, and 10-20% will maintain elevated ALT and HBV-DNA levels, thus remaining in an immunoreactive phase[15,18].

Phase 3: Previously called the "inactive carrier phase," this phase is characterized by low or undetectable levels of viral DNA, normal ALT levels, and the presence of anti-HBe in the serum. About 10-30% of patients who have entered an inactive hepatitis B state may experience reactivations of anti-HBe-positive hepatitis, marked by an increase in both ALT and HBV-DNA levels[15,18].

Phase 4: Formerly known as the "HBeAg-negative chronic hepatitis phase," this phase is characterized by the absence of HBeAg in the serum, the presence of anti-HBe, persistent or fluctuating HBV-DNA and ALT levels, and necroinflammation/fibrosis as evidenced by liver histology. Most of these individuals have viral variants with mutations in the core and pre-core regions of the HBV genome, which impair or abolish HBeAg expression[15,18].

Phase 5: This phase is characterized by negative serum HBsAg, positive antibodies to HBcAg (anti-HBc), with or without detectable anti-HBs. Known as "occult HBV infection," patients typically have normal ALT levels and usually undetectable HBV-DNA in the serum. However, viral cccDNA can be observed in the liver[16,19].

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	Chronic hepatitis	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 and therapeutic indications, according to the recent "EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection." The figure was kindly provided by Prof. M. Levrero (Oral presentation, 2018).

2. Therapies and therapeutic strategies

2.1 Current Therapeutic Strategies: Pegylated Interferon Alpha and Nucleos(t)ide Analogues

Chronic HBV infection is a global health issue, affecting over 240 million people worldwide and causing approximately 600,000 deaths annually due to disease complications[11]. The available therapies for chronic hepatitis B aim to achieve prolonged inhibition of viral replication, indeed the primary goal is to prevent the development of cirrhosis, hepatocellular carcinoma, and liver failure[20]. Complete eradication of the virus is challenging due to HBV's tendency to integrate into the host genome or remain latent as cccDNA within the nuclei of infected hepatocytes[21].

Currently, two categories of drugs have been approved: pegylated interferon alpha (PEG-IFNalpha) and nucleos(t)ide analogues (NUCs) (Figure 6). Interferons are a large family of low molecular weight (15-30 kDa) cytokines with potent immunomodulatory, antiproliferative, and antiviral activity. In the context of HBV, the antiviral activity of PEG-IFNalpha results from a complex mode of action that includes the activation of natural killer (NK) cells, inhibition of viral genome transcription through epigenetic regulation of cccDNA, destabilization of the viral nucleocapsid, and degradation of cccDNA via the activation of APOBEC3A in infected cells[22]. Pegylated interferon is administered through subcutaneous injection weekly for twelve months, thus the treatment duration is limited. However, its use is hampered by high costs and numerous associated side effects.

On the other hand, NUCs (lamivudine, telbivudine, adefovir, entecavir, and tenofovir) inhibit viral polymerase activity in chronically HBV-infected hepatocytes, leading to reduced virion production, decreased recycling of viral nucleocapsids to the nucleus of infected cells and a decline in viral cccDNA, though this is observed only after many years of treatment. NUCs do not inhibit de novo formation of cccDNA in newly infected cells, implying that persistent residual viremia during antiviral therapy may lead to the infection of new hepatocytes. The main disadvantage of this treatment is its duration; it can be discontinued without contraindications only after anti-HBs seroconversion[16], thereby avoiding HBV reactivation[23]. Since the production of anti-HBs occurs slowly in chronic infection, most patients undergo this type of therapy for life, resulting in high costs for national healthcare systems and potential lifelong side effects.

Despite both drug types being highly effective at inhibiting viral replication, for most patients, current therapies do not result in a complete cure as they do not eliminate cccDNA. Therefore, there has been significant interest in determining whether a combination of these two drugs could achieve greater therapeutic efficacy. Conceptually, combining the two therapies might lead to better HBV control due to the potential synergistic effects of their different mechanisms of action. The results of studies are inconsistent: some show significant improvement in terms of HBsAg loss and conversion to anti-HBs, while others provide evidence of the ineffectiveness of combining the two drugs[24].

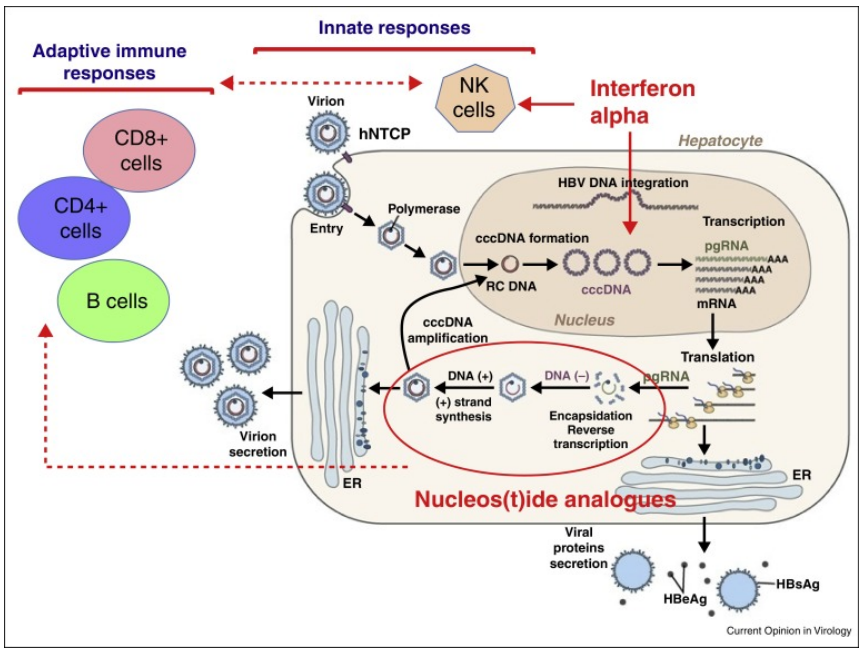

Figure 6. Action mechanism of current antiviral drugs for chronic HBV infection. Interferon-α induces the activity of NK cells and down-regulates the activity of HBV-specific cells. In experimental models, its antiviral effect has been attributed to the repression of viral transcription of cccDNA and the partial elimination of its pool. Nucleoside analogues (NUCs) block the reverse transcriptase activity of DNA polymerase, thereby reducing circulating viral DNA. Prolonged viral suppression achieved with NUCs induces the restoration of HBV-specific T lymphocytes. Both drugs do not eliminate viral cccDNA from infected hepatocytes. Source: Zolium 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 limitations of antiviral treatments have led to the necessity of exploring new therapies with the goal of reducing administration times, the risk of developing drug resistance, and avoiding potential side effects. The aim is to achieve complete elimination of HBsAg and, whenever possible, complete viral eradication. Indeed, the recent development of innovative in vitro and in vivo systems has opened the possibility to study the complex network of interactions that HBV establishes with host cells during infection, thereby identifying new potential targets for antiviral immunotherapies or vaccinations[25]. New strategies to eradicate HBV include direct-acting antivirals that focus on different stages of the HBV lifecycle, along with immune modulators designed to enhance the specific immune response against HBV or to alleviate immune blockade[26].

2.2.1 Direct-acting antivirals

Recent studies have focused on identifying compounds that can target various stages of the HBV life cycle. Key therapeutic targets being explored include blocking HBV entry into hepatocytes, degrading the viral nucleocapsid, directly targeting cccDNA, and preventing HBsAg release. Entry inhibitors like bulevirtide work by blocking HBV from entering hepatocytes, showing improved HBsAg reduction when combined with pegIFN-α, although many patients experienced virologic relapse after stopping treatment. Additionally, capsid assembly modulators (CAMs), which disrupt capsid formation and disassembly, effectively reduce HBV DNA and pgRNA levels when used with nucleos(t)ide analogs, though their impact on HBeAg and HBsAg remains limited. Post-transcription inhibitors, such as siRNAs and ASOs, can significantly lower HBsAg and may act as immune modulators, with the ASO bepirovirsen demonstrating substantial HBsAg reduction at higher doses. Finally, also nucleic acid polymers, which block the release of subviral particles from hepatocytes, are under investigation as a potential HBV treatment (Figure 7).

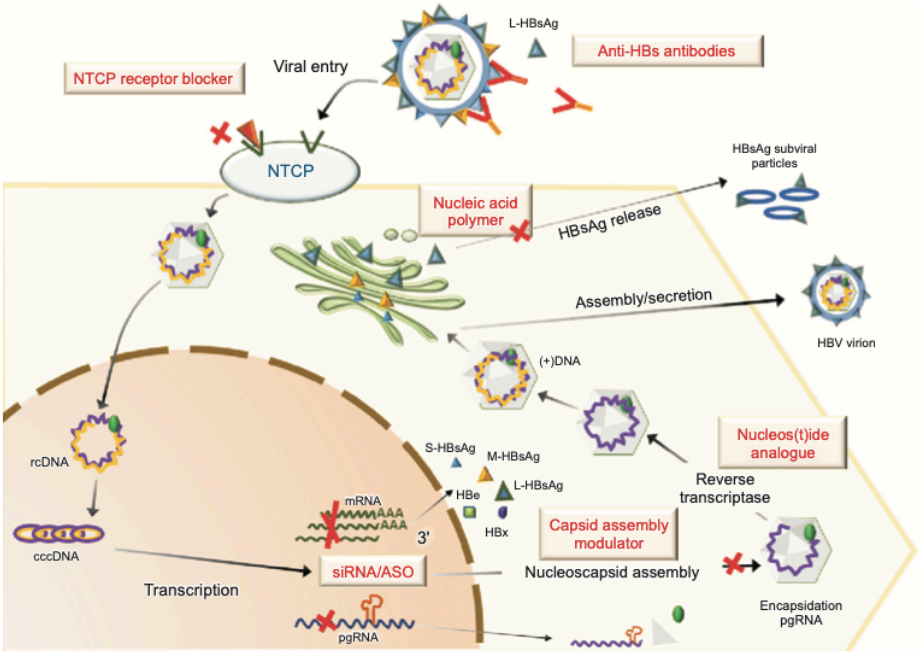

Figure 7. Hepatitis B virus (HBV) lifecycle and targets for direct-acting antiviral drugs. Source: Lok ASF. Toward a Functional Cure for Hepatitis B. Gut Liver. 2024 Jul 15;18(4):593-601. doi: 10.5009/gnl240023. Epub 2024 Mar 27. PMID: 38533651; PMCID: PMC11249939

2.2.2 Immune modulators

During HBV infection, the innate immune system is poorly activated due to the virus's ability to evade recognition, although viral replication is effectively suppressed by this system. Therefore, strategies aimed at activating different components of innate immunity and achieving localized production of antiviral cytokines in the liver have been explored as potential immunological therapies for HBV[27]. Recent studies highlight the role of TLR agonists, particularly TLR7 and TLR8, in boosting the innate immune response[26]. These agonists can partially activate immune pathways, which may improve the body's response to HBV[26]. However, their effectiveness in clinical settings has been somewhat limited, especially regarding achieving the desired loss of hepatitis B surface antigen (HBsAg). For instance, combining a TLR7 agonist with direct-acting antivirals or nucleos(t)ide analogues has shown some promise in reducing HBsAg levels. Nevertheless, this approach has still proven less effective than combinations involving pegIFN-α[26].

Furthermore, the importance of T cells in controlling viral replication has led to the development of strategies designed to enhance virus-specific T cell responses in chronic HBV patients. Two distinct strategies have been devised: the first focuses on boosting the deficient

virus-specific T cells still present, and the second involves the transfer of new virus-specific T cells into patients[27]. Therapeutic vaccination also presents a promising approach to HBV eradication. A therapeutic vaccine could induce a potent CD4+ T cell response, activate humoral immune responses, and stimulate CD8+ T cells directed against one or more HBV antigens. Various categories of vaccines are under development, including those based on recombinant HBV proteins, sub-viral particles of the envelope, and naked DNA combined with viral vectors[28]. Recent findings emphasize that combining therapeutic vaccination with other immunotherapies and direct-acting antivirals may significantly enhance the overall efficacy of HBV treatment. For instance, a phase 2b trial investigating the ChAdOx1-HBV/MVA-HBV vaccine in combination with a PD-1 inhibitor demonstrated sustained reductions in HBsAg levels among patients who had low baseline HBsAg levels[26]. Additionally, another therapeutic vaccine, BRII-179, when used in conjunction with pegIFN-α, achieved HBsAg loss in 25% of treated patients[26]. This integrated strategy could potentially lead to more durable immune responses, improving the chances of achieving a functional cure. In this context, to achieve complete T cell restoration, careful patient selection is essential to identify those who are most likely to benefit from immune modulation, representing a crucial aspect of the strategy for the new vaccine project.

3. Immune System

The immune system is a complex network of chemical mediators and cells that collaborate to defend the organism against infections. One of the main characteristics of the immune system is its ability to discriminate between what is foreign to the organism (non-self), which must be countered and, if possible, eliminated, and what is part of the organism (self), which must be preserved. Another important feature is to ensure "immunological memory." This means that if the organism encounters the same pathogen again, a very rapid response will be developed to effectively oppose it and prevent the recurrence of the pathology.

The immune system can be divided into two main branches (Figure 8).

- Innate immunity (or natural): It represents the first line of defense in individuals where the immune system has not yet developed, following an infection. This first line of defense is the oldest defense system and is common to all multicellular organisms, including insects and plants. It acts nonspecifically and rapidly, identifying a limited repertoire of antigens highly prevalent in nature. In addition to providing initial protection against infectious agents, innate immunity has the function of activating the subsequent acquired response when necessary.

- Adaptive immunity: It represents a more specific response against pathogens. This immunity operates through two different collaborating strategies, namely antibody-mediated humoral immunity and cell-mediated immunity involving the action of lymphocytes.

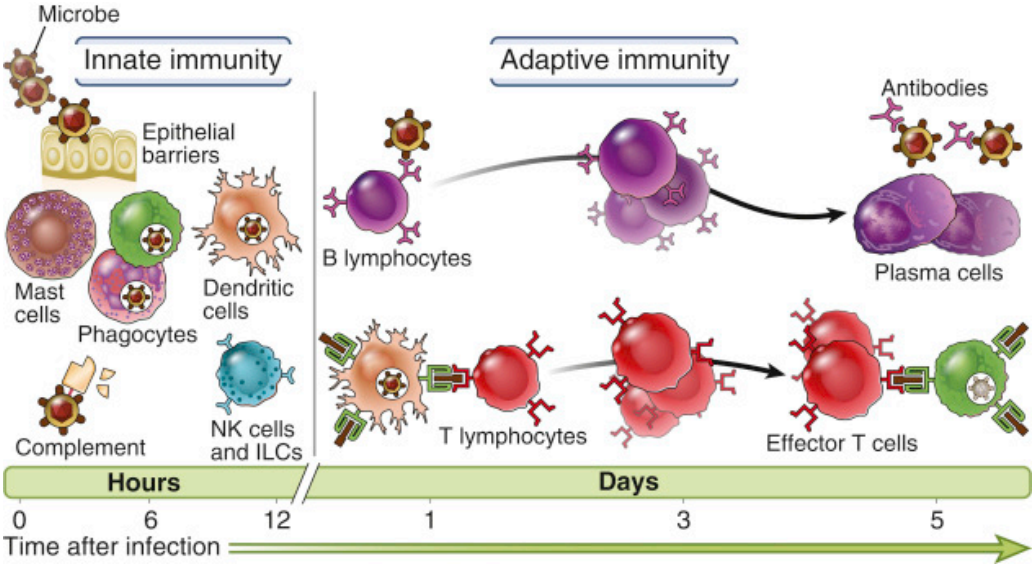

Figure 8. Innate and adaptive immunity. The mechanisms of innate immunity provide the initial defense against infections. Adaptive immune response develops later and involves the activation of lymphocytes. Source: Abbas AK, Lichtman AH: Cellular and Molecular Immunology. Ed. Elsevier, Eighth Edition. 2014.

3.1 Innate Immunity

Innate immunity is a nonspecific form of immunity present from birth, in individuals whose immune system has not yet developed and is, therefore, unable to provide specific and selective responses to pathogens. This defense mechanism relies on anatomical barriers, including the skin and mucous membranes; physiological barriers, including temperature, pH, and various soluble factors; inflammatory barriers, which are activated in response to chemical signals emitted by cells attacked by the pathogenic microorganism; and endocytosis/phagocytosis mechanisms, involving the ingestion of extracellular particles. Inflammation is one of the immune system's initial responses to infections, serving to establish a physical barrier against the spread of infection and promote the healing of any damaged tissue following pathogen clearance. The acute inflammation process is initiated by cells present in all tissues, such as macrophages, dendritic cells, Kupffer cells, and mast cells. These cells have receptors located on the surface or inside, called Pattern Recognition Receptors (PRRs), which recognize molecules widely shared by pathogens but distinguishable from host molecules, defined as Pathogen-Associated Molecular Patterns (PAMPs). At the beginning of an infection, the receptor and ligand recognition occur, leading to the release of inflammatory mediators responsible for the clinical symptoms of inflammation. Examples of PRRs include soluble mannose-binding lectin, macrophage mannose receptor, scavenger receptors, formyl

peptide receptor, and the well-studied Toll-like receptors (TLRs). TLRs are single-stranded transmembrane receptors, non-catalytic, that recognize various microbial molecules such as LPS, DNA, RNA, lipoproteins, and peptidoglycans.

3.2 Adaptive Immunity

The development of acquired immunity is supported by the action of the innate immune system and is crucial when the latter is ineffective in eliminating infectious agents. The primary functions of the acquired immune response are: the recognition of non-self-antigens, distinguishable from self-antigens; the generation of immune effector pathways specific to pathogens; and the development of immune memory, capable of rapidly eliminating a specific pathogen in the event of subsequent infections. Adaptive immune responses are the foundation for effective immunization against infectious diseases. The cells of the acquired immune system include antigen-specific T cells and B lymphocytes, which differentiate into plasma cells to produce antibodies.

Humoral immunity is mediated by macromolecules present in extracellular fluids such as antibodies, which bind to pathogens to neutralize them, preventing their entry into host cells and promoting their elimination; complement proteins, which trigger an inflammatory response; and also certain antimicrobial peptides. Antibodies are produced by plasma cells, which derive from B lymphocytes. In fact, when a foreign antigen is identified, B cells undergo proliferation and differentiation into antibody-secreting plasma cells or memory B cells. The produced antibodies then enter the bloodstream and tissues, providing effective protection against pathogens[29].

Cell-mediated acquired immunity, on the other hand, is an immune response that does not involve antibodies but primarily involves T lymphocytes, cells capable of recognizing and eliminating pathogens that survive inside phagocytes and non-phagocytic cells. T lymphocytes derive from hematopoietic stem cells found in the bone marrow and, after migrating, mature in the thymus. Each of these cells expresses a single type of receptor called the "T Cell Receptor" (TCR) and has the ability to rapidly proliferate and differentiate if it receives the appropriate signals. The TCR does not interact directly with the antigen but requires the action of cells called "Antigen-Presenting Cells" (APCs), usually dendritic cells, macrophages, B lymphocytes, fibroblasts, and epithelial cells. These cells express on their surface a group of proteins known as "Major Histocompatibility Complex" (MHC) molecules, which are classified

as either MHC class I (also called Human Leukocyte Antigen [HLA]), found on all nucleated cells, or MHC class II, found only on some immune system cells, including macrophages, dendritic cells, and B lymphocytes. Moreover, the recognition of a non-self-antigen stimulates T lymphocytes to differentiate into cytotoxic T cells (CD8+) or helper T cells (CD4+). CD8+ cells are more involved in the destruction of cells infected by foreign agents such as viruses. Their clonal expansion produces effector cells that induce apoptosis in target cells and secrete cytokines such as IFN-γ. CD4+ cells play an important role in establishing and maximizing the immune response. They do not have cytotoxic or phagocytic activity and cannot directly kill infected cells or eliminate pathogens. However, they can mediate the immune response by directing other cells to perform these tasks and regulating the type of response that develops. Activated helper T cells release cytokines that influence the activity of various cell types, with interleukin 2 (IL-2) being a key cytokine for the survival and differentiation of T lymphocytes. Additionally, these cells can further differentiate into specialized effector cells such as Th1, Th2, and Th17 cells, which fight different pathogens and also differ in the type of cytokines produced. Th1 cells, for example, are stimulated by the presence of intracellular bacteria and viruses, neutralizing them by activating macrophages, stimulating antibody production by B lymphocytes, and secreting cytokines such as IFN-γ, TNF-α, and IL-2. Th2 cells increase during parasitic infections and following stimulation by IL-4, playing a role in regulating humoral responses and releasing inhibitory cytokines such as IL-5, IL-6, and IL-10.

3.3 Immunopathogenesis of Hepatitis B Virus Infection

Liver damage resulting from HBV infection primarily depends on the interaction between the virus and the host's immune system. The virus's replication itself does not cause harm to hepatocytes; rather, the host's immune response to the infection can induce liver inflammation and damage to cells, aiming to eliminate the virus and contributing to HBV pathogenesis[30]. Although HBV has been known for many decades, its immunopathogenesis is poorly defined. The study of pathogenesis has long been limited by the lack of animal models and cell lines capable of supporting such infection[31]. Subsequently, the development of murine transgenic models has provided a better understanding of the immune system's contribution to HBV clearance.

In fact, the interaction between the virus and the immune system is responsible for viral clearance or infection progression, with the cellular immune response appearing to play a key role in determining infection resolution or, conversely, chronicity[19,32].

Initial defenses against HBV involve nonspecific mechanisms, where natural killer (NK) cells and NK-T cells play a significant role in controlling viral infection by secreting high levels of IFN-γ, leading, for example, to macrophage activation for phagocytosis. These mechanisms are activated by the release of type I interferons (IFN-α and IFN-β) by infected hepatocytes, responsible for recruiting and activating innate immune cells in the liver[33].

Complete eradication, however, cannot be achieved through innate immunity alone; effector mechanisms capable of recognizing specific viral structures are required. Thus, both humoral and cell-mediated acquired immunity come into play (Figure 9).

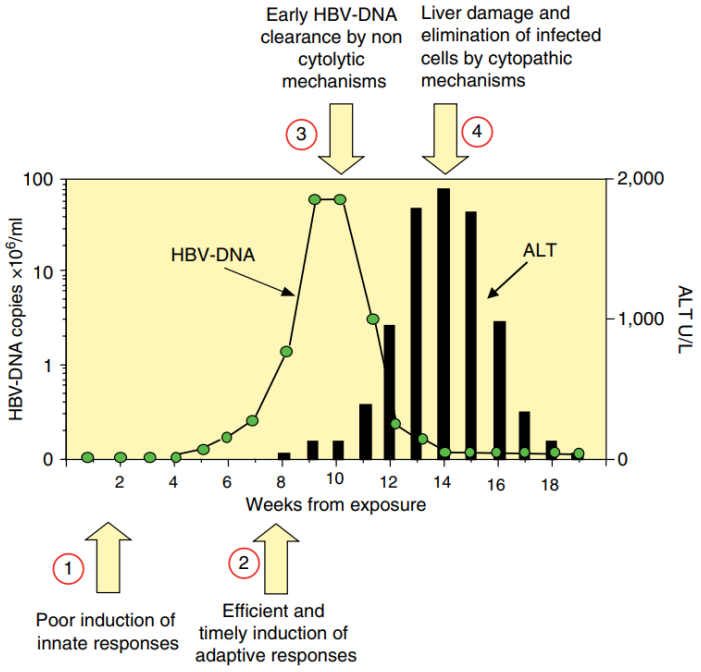

Figure 9. Sequence of immunological events after exposure to HBV. Despite the weak induction of the innate immune response by HBV, the HBV-specific T lymphocyte response is efficiently and promptly activated immediately after the onset of viral replication, which generally begins several weeks after the infection has been contracted. Source: Ferrari C, Barili V, Varchetta S, and Mondelli MU. Immune mechanisms of viral clearance and disease pathogenesis during viral hepatitis. The Liver: Biology and Pathobiology. 2020; 821-850.

Humoral immunity produces specific antibodies against viral antigens, useful for recognizing different stages of infection. HBcAg-specific IgM constitutes an early marker of infection, while anti-HBs can persist for a lifetime in individuals recovered from hepatitis, forming an

important immunological memory for protection against potential reinfections[33,34]. On the other hand, cell-mediated acquired immunity plays an important role in the infection's evolution towards resolution or chronicity (Figure 10). This immunity involves two lymphocyte populations, CD4+ and CD8+, recruited soon after viral replication begins. CD4+ cells do not have a direct effector function but are essential for activating and maintaining CD8+ T cells and virus-specific B cells[35,36]. They are also the major producers of IL-2 and IL-21, important for the differentiation of CD8+ T cells[37]. In contrast, CD8+ T cells have a direct effector function through cytotoxic mechanisms, causing the lysis of infected cells, as well as non-cytotoxic mechanisms through the secretion of cytokines such as IFN-γ and TNF-α[38,39]. The secretion of these cytokines is functional in recruiting other effector cells, such as macrophages and neutrophils, and inhibiting viral gene expression within infected hepatocytes without causing lysis[40]. This is important in limiting liver damage. CD8+ T cells play a fundamental role during infection resolution, as evidenced by the inability to undergo viral clearance in a murine model with CD8+ cell depletion[17].

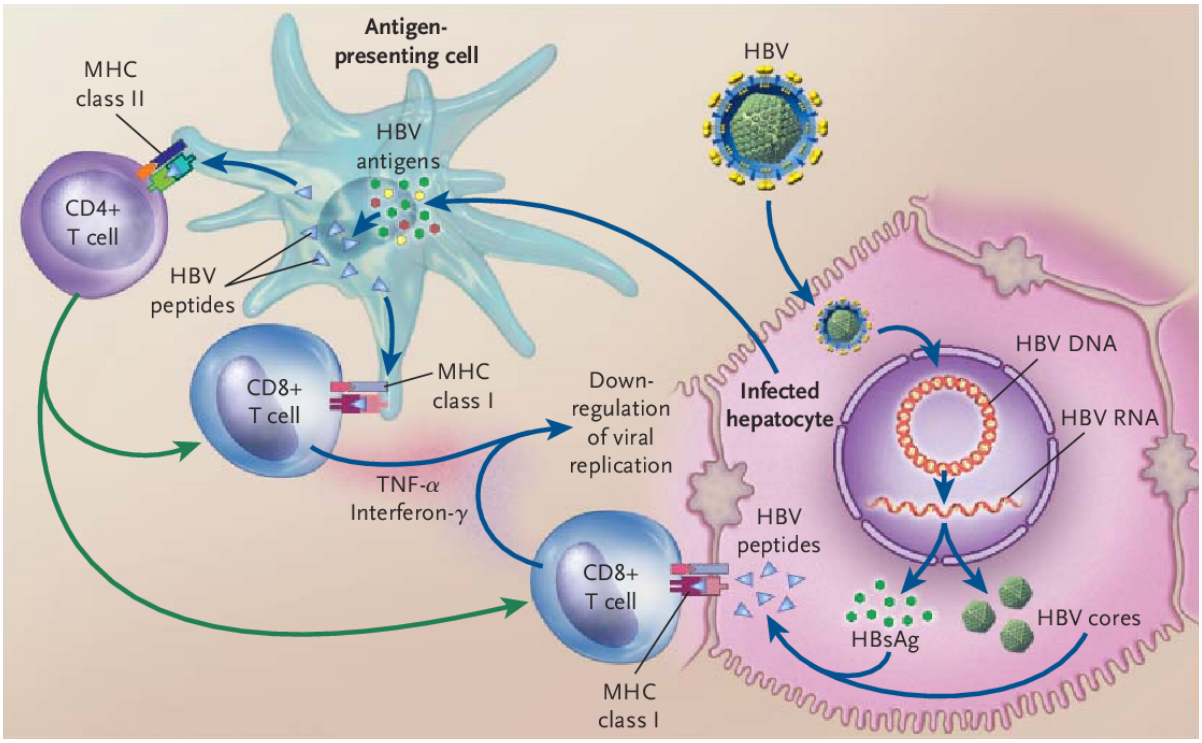

Figure 10. Cell-mediated immune response against HBV. Source: Ganem D, Prince AM. Hepatitis B virus infection—natural history and clinical consequences. New England Journal of Medicine. 2004; 350: 1118-1129.

Patients who spontaneously recover from HBV infection typically exhibit a rapid CD4 and CD8 T-cell response capable of identifying multiple viral antigenic epitopes. This ensures efficient recognition of infected hepatocytes and decreases the likelihood that HBV can evade lymphocytic control[32,41]. In contrast, patients who progress to chronicization show delayed and reduced intensity T-cell responses. Consequently, the virus has the opportunity to employ mechanisms to evade immune control, promoting infection persistence[19,42]. Furthermore, these patients display high levels of viral replication and a progressive decline in acquired immunity, with reduced levels of circulating HBV-specific T cells and decreased production of virus-specific antibodies. Indeed, following prolonged virus exposure, T cells lose their functions in a process known as "exhaustion".[33,43]

3.4 Functional exhaustion of T cells in chronic HBV infection

In chronic infections and cancer, T lymphocytes are persistently exposed to antigens and/or inflammatory signals. This leads to a functional deterioration of the involved T cells, known as the "exhaustion" phenomenon, where exhausted cells lose functionality, express multiple inhibitory receptors, and exhibit altered expression patterns[44]. Initial insights into exhaustion were gained by studying lymphocytic responses during chronic infection in mice infected with Lymphocytic Choriomeningitis Virus (LCMV)[45]. This phenomenon has also been observed in human infections such as HIV, HCV, and HBV, as well as in neoplasms. The process of T cell exhaustion is gradual, with increasing viral antigenic load resulting in the hierarchical loss of various lymphocytic functions[46]. The first stage (Partial Exhaustion I) is characteristic of early phases of chronic infections, where CD8 T cells lose the ability to produce IL-2, cytotoxic activity, and proliferative capacity. The second stage (Partial Exhaustion II) coincides with reduced production of IFN-ɣ and a lack of TNF-$\boldsymbol{\alpha}$ and IL-2 production. The penultimate stage (Full Exhaustion) involves the loss of all effector functions of CD8 T cells, culminating in the final phase of exhaustion, termed "deletion," where virus-specific T cells undergo apoptosis (Figure 11). This state, characterized by weak cytotoxic activity and reduced production of effective cytokines, is associated with prolonged expression and high levels of inhibitory receptors like PD-1, TIM-3, CTLA-4, and CD244[44,46,47]. PD-1, in particular, plays a crucial role in exhaustion, being constantly expressed in chronically infected hepatitis patients, both in

circulating virus-specific T cells and intrahepatic virus-specific T cells. The higher expression of PD-1 in intrahepatic T cells confirms a more pronounced state of exhaustion in the liver[47].

Due to the significance of inhibitory receptors, blocking these molecules in various chronic infection models has shown partial recovery of T cell responses, leading to reduced viral loads[48]. Additionally, exhaustion is influenced by the presence of immunosuppressive cytokines such as TGF-β and IL-10, whose expression increases with viral persistence, contributing to T cell dysfunction[49].

Another crucial characteristic observed in exhausted T cells is a distinct expression pattern compared to fully functional effector or memory T cells[50,51]. This includes differential expression of transcription factors such as Blimp-1, T-bet, and NFAT2. Blimp-1 and T-bet play central roles in T cell exhaustion. Blimp-1 upregulation during chronic infection induces increased expression of inhibitory receptors, while T-bet, due to persistent viral antigen stimulation, undergoes downregulation, inhibiting cellular differentiation and increasing inhibitory receptor expression[44,46]. Therefore, understanding the cellular and molecular mechanisms associated with T cell functional exhaustion can aid in identifying potential pathways for preventing or restoring T cell functionality, improving infection control. Several strategies have been employed to achieve functional recovery of exhausted T cells, including inhibitory receptor blockade, metabolic pathway manipulation, modulation of antiviral and inhibitory cytokines, and molecular reprogramming of exhausted T cells[37]. Despite promising results from early clinical studies, complete functional restoration has not been achieved. In the case of chronic HBV infections, inhibitory checkpoint blockade has led to partial recovery of functional virus-specific CD8 T cells in only a limited percentage of patients[52]. This highlights the need to identify new targets for achieving a complete restoration of T cell functionality.

Designing effective immunotherapies for chronic HBV infection requires a better understanding of the biology of virus-specific CD8+ T cells, particularly dominant antigenic targets and their phenotypic, metabolic, and functional profiles. Single-cell analyses of virus-specific T cells have revealed that these cells are not a homogenous and inefficient population. Instead, they exist as a heterogeneous population with varying levels of co-inhibitory molecules, transcription factors, terminal differentiation markers, chemokines, and other receptors[53–57]. For instance, the analysis of differential expression of T-bet and Eomes, in

combination with PD-1, has allowed the distinction of two subsets of CD8 LCMV-specific cells in chronically infected mice. Although these subsets have shown impaired function, they have demonstrated different effector activity[53]: the T-bethighEomesdimPD-1int subset represents a small population of exhausted T lymphocytes with good proliferative potential, while the TbetdimEomeshighPD-1high subset represents a numerically larger population with limited proliferative capacity and higher expression of PD-1 and other inhibitory receptors[58]. They also observed that antigen persistence leads TbethighPD-1int cells to lose T-bet expression[59], resulting in the proliferation and conversion into T-betdimEomeshighPD-1high cells, which are characterized by weak cytokine production and a poor response to PD-1 pathway blockade[53]. Recent studies have also demonstrated a key role of the transcription factor HMG-box (TOX) in inducing T cell exhaustion. TOX directly regulates the epigenetic pattern of exhausted T lymphocytes, which could represent an important target for immunotherapies aimed at improving their function[60]. In fact, the deletion of the DNA-binding domain of TOX in the LCMV murine model reduces PD-1 protein levels, resulting in increased cytokine production and more functional T lymphocytes[61]. In patients with chronic HCV infection, high TOX expression was observed in virus-specific T lymphocytes, with downregulation following antiviral treatment and complete reduction in subjects with spontaneous HCV clearance[61].

In chronic HCV infection, a T lymphocyte subpopulation characterized by a more "exhausted" phenotypic profile, TCF1-CD127-PD-1high, has been recently identified, along with a T lymphocyte subset, TCF1+CD127+PD-1high, defined as memory-like. This subset has a less "exhausted" phenotypic profile, distinguished by the co-expression of CD127 and PD-1, and is detectable even after infection resolution following antiviral therapy. It retains proliferative capacity, which is mediated by the transcription factor TCF1[56].

Limited information is available regarding HBV-specific T cell subsets in patients with hepatitis B infection. Phenotypic investigation has revealed the presence of coexisting T cell subsets with different HBV specificities (Core, Envelope, and Polymerase) and varying degrees of dysfunction[62]. Recent findings have demonstrated that CD8 polymerase-specific T cells in patients with chronically low levels of serum HBV-DNA exhibit a high degree of exhaustion, with upregulation of CD38, KLRG1, and Eomes compared to Core-specific CD8 T cells[63]. Envelope-specific T cells, on the other hand, show a notable level of exhaustion and are rarely detectable during chronic HBV infection. Finally, the anti-apoptotic marker (Bcl-2) has been

associated with different proliferative capacities in various T lymphocyte subsets in subjects chronically infected with the hepatitis B virus, suggesting the need for further investigation of its role in this disease context[63].

In summary, these results highlight the existence of distinct molecular patterns underlying different T cell subsets, associated with both different specificities for HBV and infection stages. Therefore, the study of these mechanisms can serve as a starting point for the development of innovative and personalized immunotherapies for chronic HBV infection.

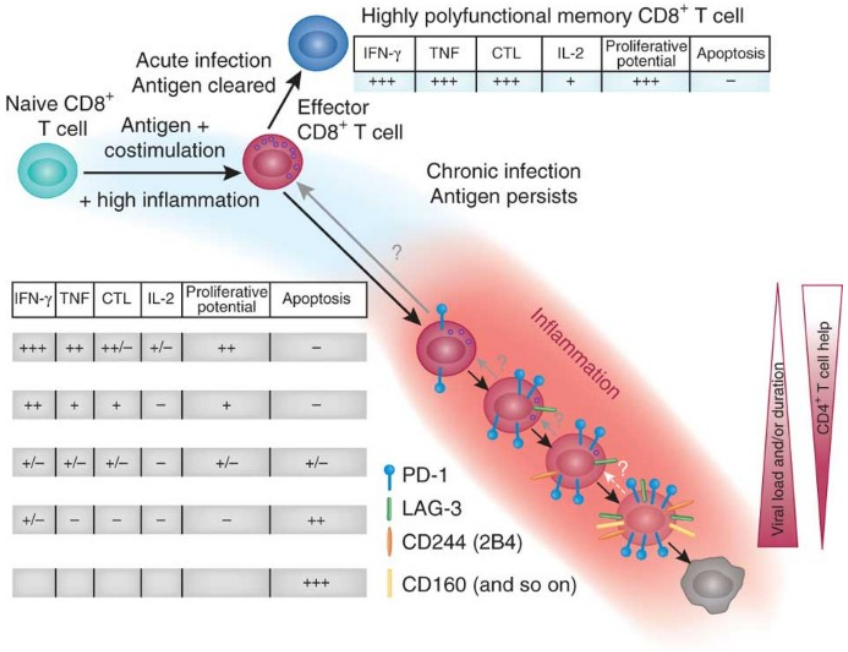

Figure 11. Hierarchical progression of the "exhaustion" phenomenon in T lymphocytes during chronic HBV infection. Difference in antiviral potential between a "healthy" effector cell and one undergoing "exhaustion," based on parameters such as cytokine production, ex vivo cytotoxic capacity, proliferative potential, and apoptosis. The first functions lost by the exhausted T lymphocyte are IL-2 production and proliferative capacity; as exhaustion progresses, all other T cell functions are gradually lost, eventually leading to apoptosis. Source: Wherry EJ. T cell exhaustion. Nature Immunology, 2011

BACKGROUND AND AIM OF THE STUDY

Chronic hepatitis B virus (HBV) infection is one of the leading causes of cirrhosis and hepatocellular carcinoma worldwide, both of which are liver diseases with a significant socio-economic impact[16]. Patients with chronic HBV infection exhibit functional defects in HBV-specific T cell responses, which result in an ineffective antiviral immune control, thereby contributing to HBV persistence[64]. Persistent exposure to high concentrations of antigen is one of the main causes of the progressive loss of T cell functionality[44]. Given the low rates of HBsAg clearance and anti-HBs seroconversion achieved with current available therapies, there is an urgent need for innovative therapeutic approaches that can accelerate HBsAg loss and promote anti-HBs antibody production[16].

Available data indicate that T cell exhaustion is characterized by a progressive and hierarchical loss of effector functions, as well as transcriptional, epigenetic, and metabolic alterations[44,49]. Additionally, it involves the upregulation of co-inhibitory receptors (PD-1, CTLA-4, TIM-3, 2B4), which can suppress T cell function after interacting with their respective ligands on the surface of antigen-presenting cells[44,49,64,65]. However, the use of molecules capable of blocking inhibitory checkpoints in vitro has only led to a partial recovery of antiviral functionality in virus-specific CD8 T cells, highlighting the need for further identification of molecular targets to develop more effective immunomodulatory therapeutic strategies[64]. Recent transcriptomic studies conducted in our laboratory have shown a predominant downregulation of genes involved in various cellular functions, with a particularly significant impact on mitochondrial processes and the ubiquitin-proteasome system[65]. Moreover, it has been demonstrated that compounds with selective antioxidant activity targeting the mitochondria can significantly restore the functionality and viability of HBV-specific T cells[65].

According to studies conducted in the murine model of chronic Lymphocytic Choriomeningitis Virus (LCMV) infection and in chronic HCV and HIV infections in humans, the analysis of co-expression of transcription factors such as T-bet, Eomes, and TCF-1, in association with differentiation markers and co-inhibitory receptors (CXCR5, CD127, and PD-1), has revealed that virus-specific T lymphocytes represent a heterogeneous cell population. This population

is characterized by the presence of distinct T cell subpopulations with varying degrees of exhaustion and different responses to inhibitory pathway blockades, coexisting within a single infected patient[44,56]. Similarly, during chronic HBV infection, the HBV-specific CD8 T cell population is not uniformly represented[66]; however, the currently available information on this topic is limited.

Based on this background, the aim of our study was to characterize in better detail the functional heterogeneity of HBV-specific and total CD8 T cells in chronic hepatitis B (CHB) patients, to assess whether this can affect individual responses to immune modulatory strategies and to identify CD8 T cell functional and phenotypic parameters to quantify HBV-specific T cell impairment in individual patients in the perspective of novel diagnostic tools based on T cell analysis[67].

The information presented in this thesis has been previously published in the article: Rossi M, Vecchi A, **Tiezzi C**, et al. Gut 2023;72:2123–2137[67]

MATERIALS AND METHODS

1. Patient populations

A total of 376 CHB patients were enrolled and screened for HLA-A2 expression. Patients were enrolled at the "Unit of Infectious Diseases of Parma", at the "Unit of Internal Medicine of the Modena University Hospital" and at the "Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico", Milan. A total of 160 resulted HLA-A2+ and 60 of them showed detectable frequencies of $core_{18\text{-}27}$ dextramer+CD8T cells (Figure 12A). Seventy-one HLA-A2+patients were tested also with a polymerase-specific dextramer containing the pol sequence 455–463 and 23 of them came out to be positive. All patients who showed the presence of at least one of the two tested CD8 T cell epitopes (64 patients) represent the final HLA-A2+ study population, which comprises the following categories of patients (Table 2):

- Thirty-five treatment-naïve patients (CHB).
- Sixteen immune subjects spontaneously recovered from chronic HBV infection (spontaneous seroconversion, SS).
- Thirteen NUC resolved patients, as shown by HBsAg clearance following NUC therapy (NUC-RES).

Seven healthy subjects (H) were enrolled as controls.

Additional 24 HLA-A2 negative treatment-naïve CHB patients were enrolled and studied to further validate the association between phenotype of total CD8 T cells and T cell responsiveness to modulatory compounds (Table 3).

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Table 2. Demographic and clinical details of different HLA-A2+ patient categories

	Patient ID	Gender	Age (years)	Genotype	Therapy	HBsAg (IU/ml)	anti HBs (UI/ml)	HBeAg (IU/ml)	ALT (IU/L)	HBV-DNA (UI/ml)	Detectable CD8+ T cell response	
											$Core_{18-27}$	$Pol_{455-463}$
CHB	CHB 1	M	68	D	naive	23549	-	-	52	14840	+	-
	CHB 2	M	35	D	naive	1932	-	-	136	237528	+	-
	CHB 3	M	36	B	naive	3472	-	-	607	8132680	+	+
	CHB 4	F	51	D	naive	482	-	-	43	66400	+	-
	CHB 5	F	40	D	naive	5398	-	-	168	842528	+	-
	CHB 6	F	57	A	naive	4600	-	-	65	229402	+	-
	CHB 7	M	54	D	naive	694	-	-	183	9856	+	-
	CHB 8	F	40	D	naive	608	-	-	93	881000	+	n.d.
	CHB 9	F	43	D	naive	714	-	-	30	11453	+	-
	CHB 10	M	34	D	naive	4085	-	-	85	3497520	+	n.d.
	CHB 11	M	44	D	naive	8819	-	-	49	20700	+	+
	CHB 12	M	46	n.d.	naive	+	-	-	133	n.d.	+	+
	CHB 13	M	64	A	naive	185	-	-	33	5140	+	-
	CHB 14	F	29	D	naive	5139.73	-	-	21	31721	+	-
	CHB 15	F	57	D	naive	19679	-	-	34	509000	+	+
	CHB 16	M	34	D	naive	2752	-	-	40	86620	+	n.d.
	CHB 17	F	42	C	naive	144	-	+	51	6800000	+	+
	CHB 18	M	54	D	naive	4775	-	-	68	153246	+	-
	CHB 19	M	71	D	naive	n.d.	-	-	165	260000	+	n.d.
	CHB 20	M	52	D	naive	2662	-	-	40	21331	+	-
	CHB 21	M	44	D	naive	3736	-	-	146	1400591	+	-
	CHB 22*	F	66	D	naive	98	148	-	23	7744	+	-
	CHB 23	F	26	A	naive	7171.45	-	-	12	3380	+	+
	CHB 24	M	50	D	naive	2940	-	-	35	9733	+	-
	CHB 25	M	45	n.d.	naive	10539.72	-	+	123	999848	+	-
	CHB 26	M	38	D	naive	5031.25	-	-	57	110009	+	-
	CHB 27	M	27	E	naive	30915.9	-	-	41	23337	+	-
	CHB 28	M	45	D	naive	1764	-	-	16	7760	+	+
	CHB 29	M	71	D	naive	3097	-	-	23	5030	+	+
	CHB 30	M	44	D	naive	0.57	-	-	33	283	+	+
	CHB 31	M	28	D	naive	35784	-	-	55	34714	+	-
	CHB 32	F	60	D	naive	2190	-	-	19	14400	+	-
	CHB 33	F	42	D	naive	12539	-	-	90	31836000	-	+
	CHB 34	M	61	A	naive	1271.09	-		30	438	+	-
	CHB 35	M	47	D	naive	15282	-	-	41	4456	-	+
NUC resolved	NUC-RES 1	M	81	n.d.	-	-	33	-	14	-	+	n.d.
	NUC-RES 2	M	57	D	-	-	26	-	28	-	+	n.d.
	NUC-RES 3	M	38	D	-	-	190	-	19	-	+	+
	NUC-RES 4	F	46	F	-	-	173	-	20	-	+	+
	NUC-RES 5	M	64	A	-	-	325	-	23	-	+	n.d.
	NUC-RES 6	M	79	D	-	-	4	-	19	-	+	n.d.
	NUC-RES 7	M	43	A	-	-	506	-	17	-	+	+
	NUC-RES 8	M	60	n.d.	-	-	2	-	31	-	+	-
	NUC-RES 9	M	36	n.d.	-	-	216.91	-	23	-	+	+
	NUC-RES 10	M	48	n.d.	-	-	> 1000	-	29	-	+	-
	NUC-RES 11	M	65	n.d.	-	-	n.d.	-	32	-	+	+
	NUC-RES 12	M	65	D	-	-	2	-	19	-	+	+
	NUC-RES 13	M	62	n.d.	-	-	189.55	-	35	-	-	+
Spontaneous Seroconversion	SS 1	M	59	n.d.	-	-	45	-	30	-	+	-
	SS 2	F	69	n.d.	-	-	66	-	26	-	+	+
	SS 3	M	49	n.d.	-	-	+	-	36	-	+	n.d.
	SS 4	M	60	n.d.	-	-	270	-	31	-	+	n.d.
	SS 5	F	73	n.d.	-	-	7	-	15	-	+	n.d.
	SS 6	F	41	n.d.	-	-	451	-	11	-	+	n.d.
	SS 7	M	41	n.d.	-	-	+	-	20	-	+	n.d.
	SS 8	M	38	n.d.	-	-	n.d.	-	24	-	+	+
	SS 9	M	58	n.d.	-	-	47	-	48	-	+	n.d.
	SS 10	F	59	n.d.	-	-	+	-	12	-	+	+
	SS 11	M	74	n.d.	-	-	3	-	15	-	+	+
	SS 12	M	60	n.d.	-	-	59	-	12	-	+	-
	SS 13	F	59	D	-	-	20.11	-	16	-	+	-
	SS 14	F	50	D	-	-	2	-	36	-	+	n.d.
	SS 15	F	67	n.d.	-	-	11.2	-	19	-	+	-
	SS 16	M	69	n.d.	-	-	70.67	-	19	-	-	+
Healthy	H1	F		-	-	-	-	-	n.d.	-	Flu matrix	
	H2	M		-	-	-	-	-	n.d.	-	Flu matrix	
	H3	F		-	-	-	-	-	n.d.	-	Flu matrix	
	H4	F		-	-	-	-	-	n.d.	-	Flu matrix	
	H5	F		-	-	-	-	-	n.d.	-	Flu matrix	
	H6	F		-	-	-	-	-	n.d.	-	Flu matrix	
	H7	M		-	-	-	-	-	n.d.	-	Flu matrix	

n.d. = not done

*Patient followed for >10 years; CHB diagnosed by liver biopsy; fluctuations of ALT (from normal to slightly elevated) and viremia (from 5,000 IU/ml to 40,000 IU/ml) levels; recent detection of anti-HBs combined with HBsAg.

Horizontal dashed line (–) indicates a wild-type residue, whereas amino acid substitutions are shown in bold.

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Table 3. Demographic and clinical details of HLA-A2- CHB patients

	Patient ID	Gender	Age (years)	Genotype	Therapy	HBsAg (IU/ml)	anti HBs (UI/ml)	HBeAg (IU/ml)	ALT (IU/L)	HBV-DNA (UI/ml)
CHB	CHB 36	M	63	D		996	-	-	58	76222
	CHB 37	F	67	A		15970	-	-	42	9856
	CHB 38	M	20	n.a		3761	-	-	24	246
	CHB 39	F	42	D		7921	-	-	42	121774
	CHB 40	F	52	n.a		13945	-	-	86	1848322
	CHB 41	M	43	D		5459	-	-	113	1180000
	CHB 42	M	48	D		17589	-	-	50	3880200
	CHB 43	M	81	n.a		11695	-	-	116	2866690
	CHB 44	M	37	D		182	-	-	27	11995
	CHB 45	F	44	D		691	-	-	31	10120
	CHB 46	M	37	D		65626	-	-	26	169601
	CHB 47	F	61	D		3865	-	-	142	234140
	CHB 48	M	54	D		18436	-	-	55	1890237
	CHB 49	M	50	C		2028	-	-	222	12030348
	CHB 50	M	50	D		11557	-	-	24	12102
	CHB 51	M	36	D		7458	-	-	25	47946
	CHB 52	F	29	D		30606	-	+	59	1,35E+08
	CHB 53	M	23	D		9329	-	-	26	5837
	CHB 54	M	59	D		55988	-	+	72	3,49E+08
	CHB 55	F	71	D		231	-	-	25	280155
	CHB 56	F	46	D		662	-	-	19	3140
	CHB 57	M	64	D		228	-	-	19	116997
	CHB 58	M	29	n.a		1223	-	-	54	2050
	CHB 59	M	47	D		8181	-	-	212	150300

The information presented in this thesis has been previously published in the article: Rossi M, Vecchi A, **Tiezzi C**, et al. Gut 2023;72:2123–2137[67]

2. Immunological analysis

2.1 Peptides and dextramers

To evaluate virus-specific CD8 T cells responses in HLA-A2 positive patients, peptides corresponding to the HLA-A2-restricted core 18–27 (FLPSDFFPSV) and polymerase 455– 463 (GLSRYVARL) epitopes of genotype D HBV were used. As a control, a peptide containing the influenza A virus (FLU) matrix (GILGFVFTL) HLA-A2-restricted epitope was employed. Peptides were purchased from Proimmune (Oxford, UK). The PE-labeled dextramer peptide-HLA class I complexes corresponding to HBV-core and FLU matrix were purchased from Immudex (Copenhagen, Denmark). PBMC were also stimulated with a panel of 15-mer peptides, overlapping by 10 residues, covering the HBV core and polymerase genotype D sequences, pooled in 3 mixtures (core, pol 1, pol 2), as previously described[65].

2.2 Phenotypic analysis of HBV-specific CD8 T cells

PBMCs were isolated from fresh heparinized blood by Ficoll-Hypaque density gradient centrifugation and cryopreserved in liquid nitrogen until the day of analysis. To define the stage of CD8+Tcell differentiation, dextramer+CD8+ cells were stained with antibodies to cytokine receptors (CD127), anti-apoptosis/cell survival (Bcl-2 and TCF-1) and T cell exhaustion (PD-1, TOX and CD39) markers. All determinations were performed using LRS Fortessa (Becton Dickinson, BD, Immunocytometry System, CA, USA). The data were processed with the FACS-DIVA or Flow-Jo software (BD, Becton Dickinson).

2.3 Ex vivo functional assessment of HBV-specific CD8 T cells

While viral peptides represent the most physiologically-relevant stimulus to study antigen specific CD8 T cell responses, peripheral HBV-specific CD8 T cells in CHB patients typically show a very limited response to HBV peptides ex vivo. Therefore, we performed preliminary experiments to evaluate various stimuli acting at different levels of the T cell signaling cascade (anti-CD3/anti-CD28, PMA/ionomycin, IL2, IL12). Based on these studies (data not shown), we selected PMA/ionomycin stimulation for functional T cell characterization experiments. After one-hour PBMC stimulation with PMA (phorbol 12-myristate 13-acetate, 100ng/ml) and ionomycin (1µg/ml), brefeldin-A (BFA, 10 µg/ml, BD, Becton Dickinson) was added for the last 3 hours. Then, cells were washed and surface-stained with dextramer-PE and fluorochrome-

conjugated antibodies. PBMC were fixed, permeabilized and stained with cytokine antibodies according to the manufacturer's instructions. Samples were acquired on a BD LSR Fortessa and analyzed with the FlowJo software. Data were expressed as the total frequency of IFN-γ, TNF-α single positive and IFN-γ/TNF-α double positive dextramer+ CD8 T cells.

2.4 'z-Score' and 'Exhaustion index'

Median fluorescence intensity (MdFI) values of exhaustion and memory markers (PD-1, TOX, CD39 and CD127, Bcl-2, TCF-1, respectively) expressed by HBV-specific CD8 T cells were standardised by calculation of the z-Score to overcome the problem raised by the different order of magnitude of MdFI values detected for individual parameters.

All z-Scores of each patient's MdFI were calculated relative to reference populations represented by NUC-RES and SS, using the formula z = (x-m)/s where "z" is the Z-score corresponding to the value x in the original set, "m" is the mean of the original set, and "s" is the standard deviation of the original set of data. A new variable, named Exhaustion Index (EI), was then created by averaging PD-1, TOX, CD39, CD127, Bcl-2 z-Scores: EI=mean (z-PD-1 + zTOX + z-CD39 – z-CD127 – z-Bcl-2). This new variable takes into account the contribution of each MdFI value irrespective of its original scale. A threshold of 2 (like in the gaussian case) was choosen as cut-off to separate patients with high vs low EI. Calculations were performed with the statistical package IBM-SPSS v.26.

2.5 In vitro T-cell expansion and treatment with immunomodulators

Short-term T-cell lines were generated by 8-10 days PBMCs stimulation either with the core18-27 peptide (1 μM) or with a panel of 187 15-mer peptides (1 μM), overlapping by 10 residues, covering the HBV core and polymerase genotype D sequence, pooled in 3 mixtures (core, pol 1, pol 2) alone or in the presence of the different tested compounds, including the polyphenolic compound Trans-Resveratrol (5 or 10 μM, Sigma-Aldrich, Missouri, USA), the mitochondria-targeted antioxidant MitoTempo (10 or 100 μM, Sigma-Aldrich), an anti-PD-L1 antibody (5 or 10 μg/ml, Invitrogen, Clone MIH1), a small PD-L1 inhibitor molecule (concentrations of 0.5-5 μM, PD-L1 SM, GS-418, kindly provided by Gilead Sciences, Inc., Foster City, CA) and a selective TLR8 agonist (concentrations of 0.5-10 μM, Selgantolimod, provided by Gilead Sciences, Inc., Foster City, CA). In experiments with anti-PD-L1, the small

PD-L1 inhibitor molecule and the TLR8 agonist, PBMCs were pre-incubated for 45 minutes with each compound and then stimulated with the peptides (1 µM). To study the effect of mutations on the HBV-specific CD8 T cell function, PBMCs were stimulated for 10 days in vitro with WT- or variant-specific core 18-27 peptides (1 µM).

2.6 Statistical methods

The GraphPad Prism software Version 7.00 and JASP Version 0.9.2.0 were used for statistical analysis. Normality distribution of data was tested by the Kolmogorov-Smirnov test. Differences between multiple patient groups were evaluated by Kruskal-Wallis nonparametric test and the p-values were calculated and corrected for pair-wise multiple comparisons. Comparisons between two groups were assessed by the Mann-Whitney U test or the Wilcoxon-matched-paired test. Data correlations were evaluated by Spearman tests.

The suitable cut-off for the different CD8 T cell phenotypic profiles was obtained using a ROC curve analysis, where Exhaustion Index (low and high) was used as classification criterion. The optimal threshold points on the ROC curves were chosen as those maximizing the difference between true positive and false-positive rates, the so-called Youden's index. The Youden index is the farthest point on the ROC curve from the line of equality (diagonal line). AUC (area under the curve) values were all close to 1, showing very high discriminating power. Calculations were performed with the statistical package IBM-SPSS v.26.

The information presented in this thesis has been previously published in the article: Rossi M, Vecchi A, **Tiezzi C**, et al. Gut 2023;72:2123–2137[67]

RESULTS

Phenotypic heterogeneity of HBV-specific CD8 T cells in CHB

HLA-A02-restricted core$_{18-27}$-specific CD8+T cells were analysed ex vivo in 35 CHB patients before starting antiviral therapy, all with stably elevated or fluctuating viraemia and ALT levels (as typically observed in HBeAg- CHB patients), in 13 NUC treated patients after anti-HBs seroconversion and therapy suspension (NUC-RES) and in 16 subjects who became anti-HBs+following chronic HBV carriage ('SS') (Table 2 and Figure 12A). Flow cytometry analysis of the exhaustion and memory markers PD-1, TOX, CD39, CD127, Bcl-2 and TCF1[54,56,61,68] showed higher PD-1, TOX, CD39 and lower Bcl-2, CD127 expression on core18-27-specific CD8 T cells from untreated CHB patients compared with resolved subjects (NUC-RES and SS) and to influenza-specific CD8 T cells from healthy controls. Instead, TCF1 expression was similar in all patient categories (Figure 12B). A direct correlation was observed in CHB patients between individual exhaustion markers (PD-1 vs TOX, CD39 vs TOX and CD39 vs PD-1) on one hand and between individual differentiation/memory markers (Bcl-2 vs TCF1 and CD127 vs Bcl-2) on the other (Figure 12C). Instead, exhaustion and differentiation/memory markers were inversely correlated to each other (Figure 12C). Quantification of the overall level of CD8 T cell exhaustion in each patient by an exhaustion score (EI) that we generated from z-standardised MdFI values of PD-1, TOX, CD39, CD127, Bcl-2, allowed to distinguish two cohorts of CHB patients with EI values lower or greater than 2, comprising 72% (23 patients; Figure 13A) and 28% (9 patients; Figure 13A, black bars within the yellow square) of the overall CHB cohort, respectively. Combined analysis of the different exhaustion and memory markers is well recapitulated by the radar plot in Figure 13B, where blue and orange lines correspond to each z-normalised phenotypic parameter in CHB patients with high and low EI values, respectively. The high EI CHB patient group was also characterised by the presence of a unique CD8 T cell subset composed of PD-1highCD127low/- cells that was poorly represented or totally negative in the remaining group of CHB patients and completely absent in the resolver groups (NUC-RES and SS) (Figures 13C, D). As depicted also in figure 13B, a significant difference in the expression of PD-1, TOX, CD39, Bcl-2 and CD127 was detected in core$_{18-27}$-specific CD8 T cells of CHB patients with high and low EI (Figures 13E).

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In order to extend T cell analysis to another HBV antigen, CD8 T cells from patients with active (n=45) and resolved (n=26) chronic infections were also stained with the $pol_{455-463}$ dextramer. $Pol_{455-463}$-specific CD8 T cells were found only in a limited proportion of the HBV-infected chronic patients (11 of 45 tested, 24%) (Table 2 and Figure 14A). In all patient categories they were significantly less represented than $core_{18-27}$-specific CD8 T cells (Figures 14B, C, $p<0.0001$ and $p=0.0012$ in CHB and resolved patients, respectively). In line with previous studies [69–71], in chronic patients $pol_{455-463}$-specific CD8+T cells expressed higher levels of TOX and lower levels of PD-1 (Figure 14D, $p=0.0156$ and $p=0.07$, respectively) compared with $core_{18-27}$-specific CD8+T cells, while no differences in CD39, Bcl-2, TCF1 and CD127 expression were observed between CD8 cells of different specificity (Figure 14D). No significant differences were also detected in the expression of exhaustion and differentiation/memory markers between $pol_{455-463}$-specific CD8+T cells from HBV-infected chronic patients and individuals with spontaneous or NUC-induced control of infection (Figure 14E). Overall, our results suggest that $pol_{455-463}$ and $core_{18-27}$-specific CD8 T cells in CHB significantly differ with respect to peripheral blood frequencies and phenotypic parameters highlighting two distinct exhaustion profiles.

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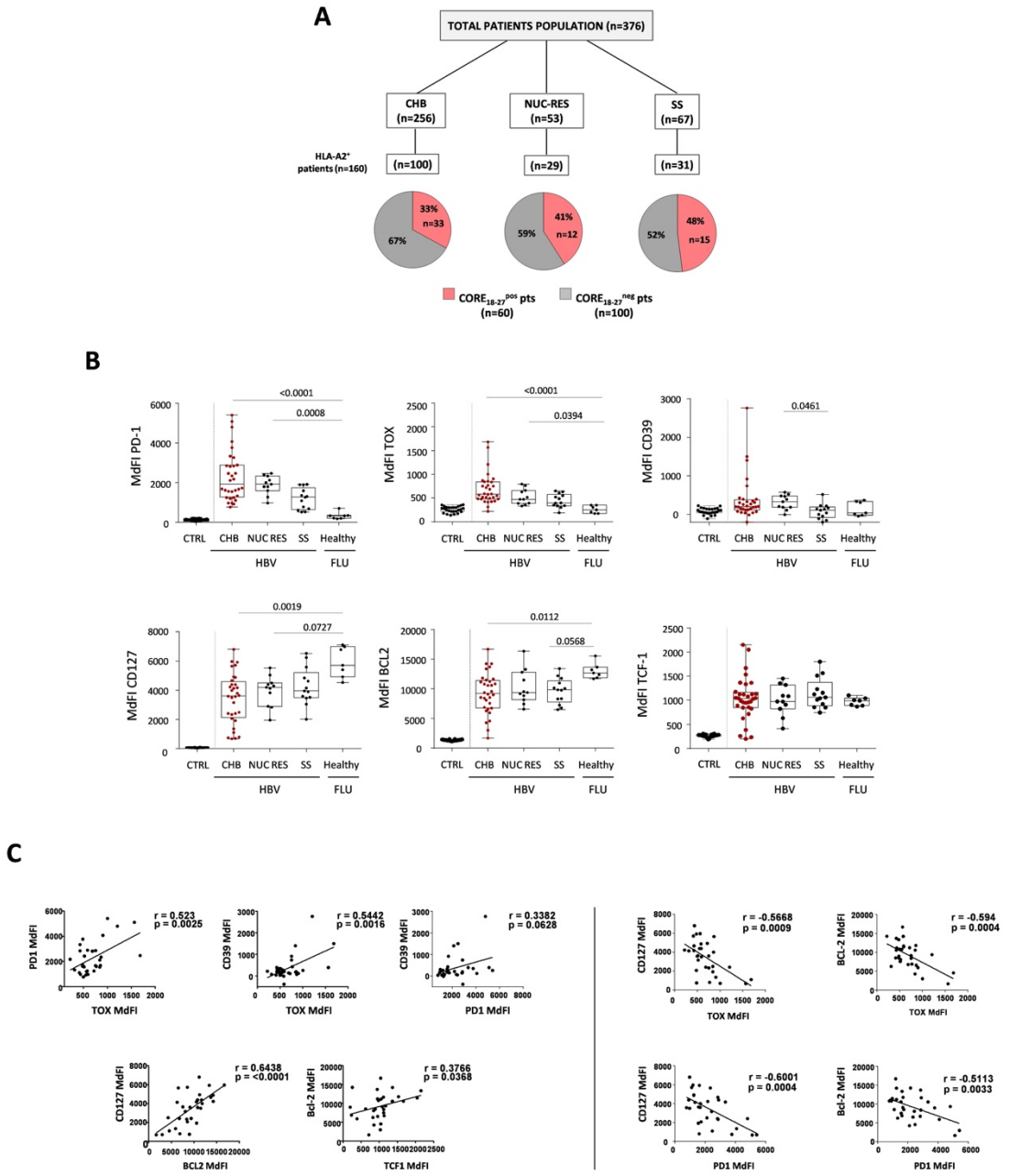

Figure 12. **Phenotypical analysis of HBV-specific CD8 T cells.** (A) Flow chart of the patient cohorts enrolled in the study. Pie plots indicate core$_{18\text{-}27}$-dextramer+ CD8 T cell frequency. (B) PD-1, TOX, CD39, CD127, Bcl-2 and TCF1 MdFI of HBV-core$_{18\text{-}27}$ and influenza-specific CD8 T cells in the indicated study groups and in the negative CD8 population calculated for each specific marker within the total CD8+ T cells (negative control). Box-whisker plots show median values and 5th/95th percentiles; each dot represents a single patient. Statistics by the Kruskal-Wallis with Dunn's correction test. (C) Correlation of exhaustion and differentiation/memory markers by core$_{18\text{-}27}$-specific CD8 T cells in CHB patients. Statistics by the Spearman's correlation test. CHB, chronic hepatitis B; HBV, hepatitis B virus; MdFI, median fluorescence intensity; NUC RES, NUC resolved patients; SS, spontaneous seroconversion.

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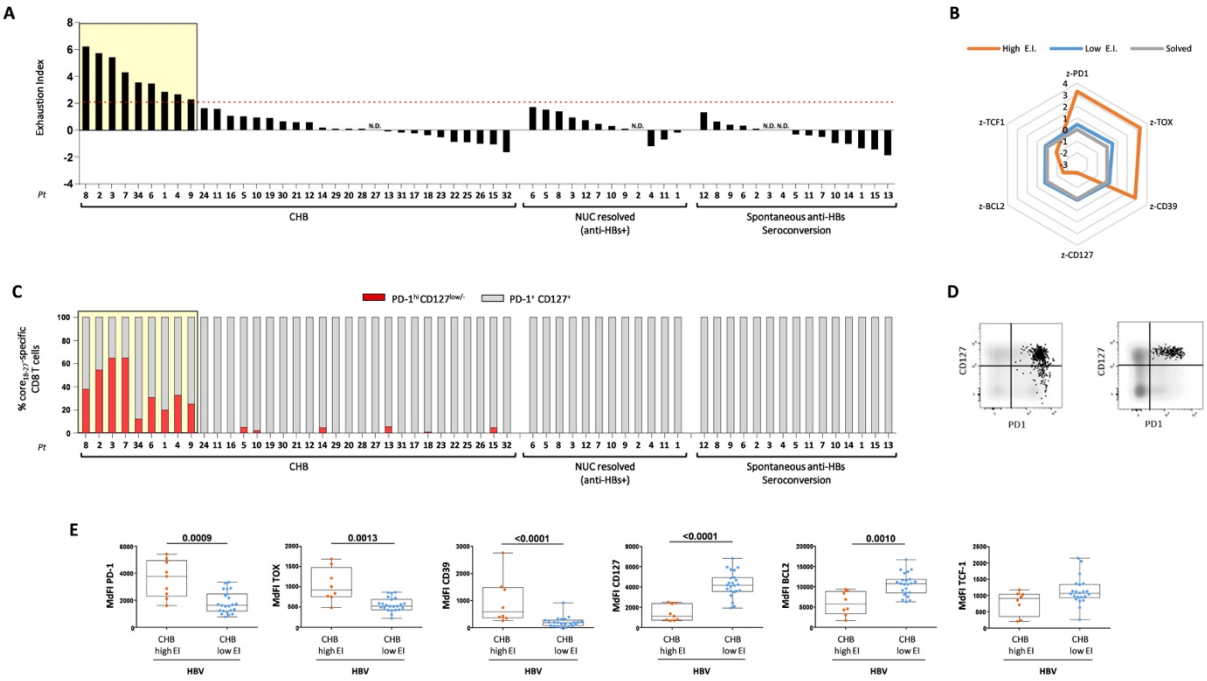

Figure 13. Classification of CHB patients according to CD8 T cells expression of exhaustion and memory/differentiation markers. (A) EI values were defined by PD-1, CD39, TOX, CD127, Bcl-2 and TCF1 expression on core$_{18\text{-}27}$-specific CD8 T cells for each study cohort. Each bar represents an individual patient. The yellow area indicates patients with EI values above 2. (B) Radar plots depict the mean z-Score values of the indicated HBV-specific CD8 T cell exhaustion and memory/differentiation markers. Blue and orange lines indicate z-Score values of patients with high or low EI values, respectively; the grey line NUC-induced and spontaneous anti-HBs seroconverters. (C) Frequencies of PD-1highCD127low/- and CD127+/PD-1+ cells among the core$_{18\text{-}27}$-specific CD8 T cell population (red and grey bars, respectively). Each bar represents an individual patient. (D) Representative FACS plots from two chronic patients with or without the PD-1highCD127low/- subset. (E) PD-1, TOX, CD39, CD127, Bcl-2 and TCF1 expression on core18-27-specific CD8 T cells of CHB patients with high and low EI values. Box-whisker plots as in figure 12; each dot represents a single patient. Statistics by the Mann-Whitney U test. CHB, chronic hepatitis B; EI, Exhaustion Index; HBV, hepatitis B virus.

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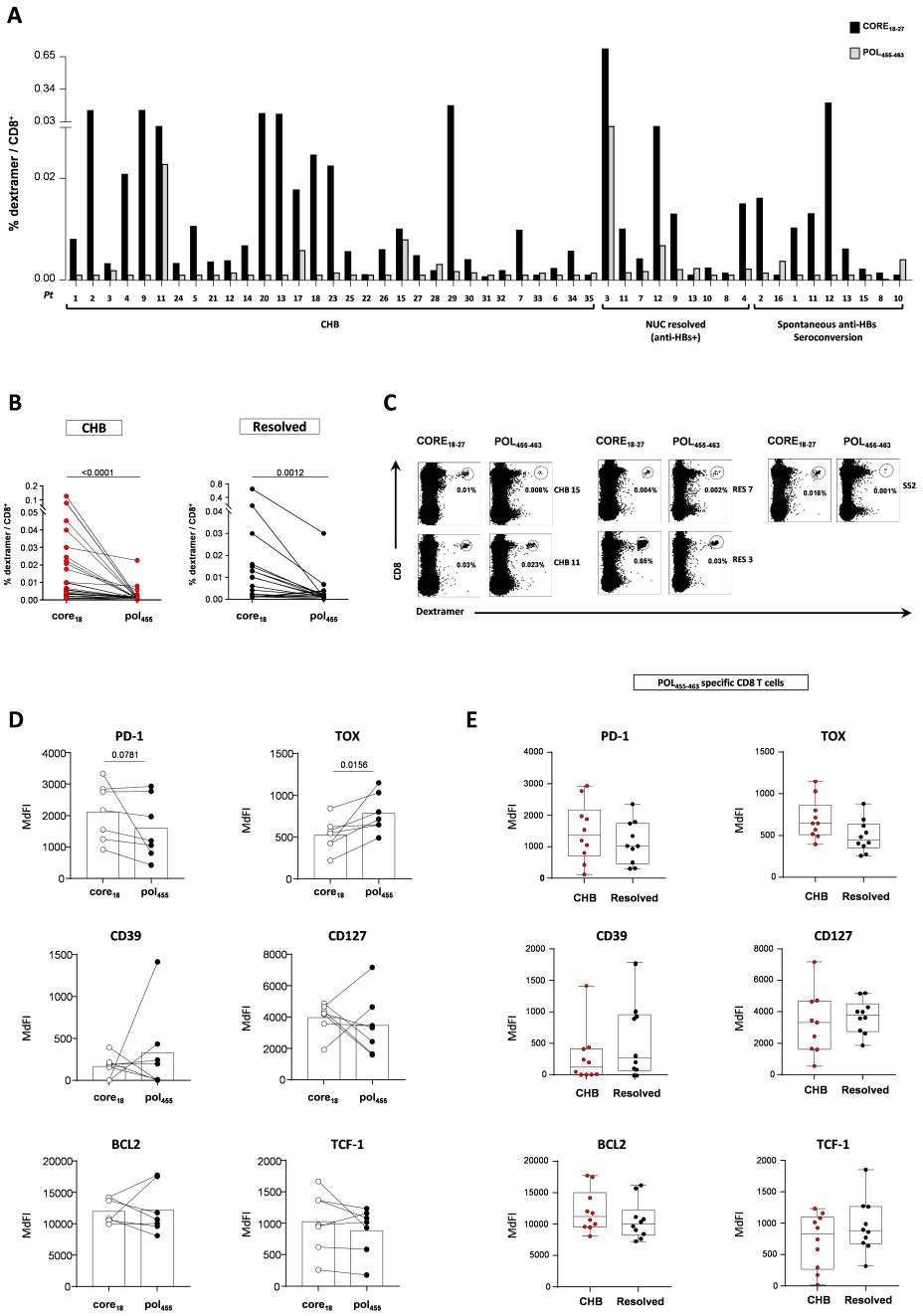

Figure 14. Different detection rate and phenotype of core18-27-specific and pol455-463-specific CD8 T cells. (A, B) Frequencies of core$_{18\text{-}27}$- and pol$_{455\text{-}463}$-specific CD8 T cells within the total CD8+T cell population from 31 CHB and 18 resolved patients who showed a detectable frequency for at least one HBV dextramer (paired core and polymerase data are illustrated in panel B). (C) Representative FACS plots from two CHB and three resolved patients. (D) PD-1, TOX, CD39, CD127, Bcl-2 and TCF1 expression within core$_{18\text{-}27}$- and pol$_{455\text{-}463}$-specific CD8 T cells from 7 CHB patients; statistics by Wilcoxon matched-pairs test. (E) PD-1, TOX, CD39, CD127, Bcl-2 and TCF1 expression on pol$_{455\text{-}463}$-specific CD8 T cells in CHB and resolved patients (n=10 for both groups). CHB, chronic hepatitis B; HBV, hepatitis B virus.

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Different CD8 T cell functionality of CHB patients with different EI values

Cytokine production by core$_{18-27}$-specific CD8 T cells was then analysed ex vivo (Figure 15A). Functionality of core-specific CD8 T cell was very variable, because some CHB patients showed cytokine production levels comparable to NUC and spontaneous anti-HBs seroconverters, while other CHB patients produced cytokines less efficiently (Figure 15B, left graph). HBV-specific CD8 T cells from CHB patients with lower EI were more efficient in cytokine production (Figure 15B, right graph) which was inversely correlated to the EI (Figure 15C). Total cytokine production by the overall HBV-specific CD8 T cell population showed a positive and negative correlation with Bcl-2 and TOX levels, respectively (Figure 15D). The same trend (but without significant levels of correlation) was observed with the other exhaustion (PD-1 and CD39) and memory (CD127, TCF-1) markers.

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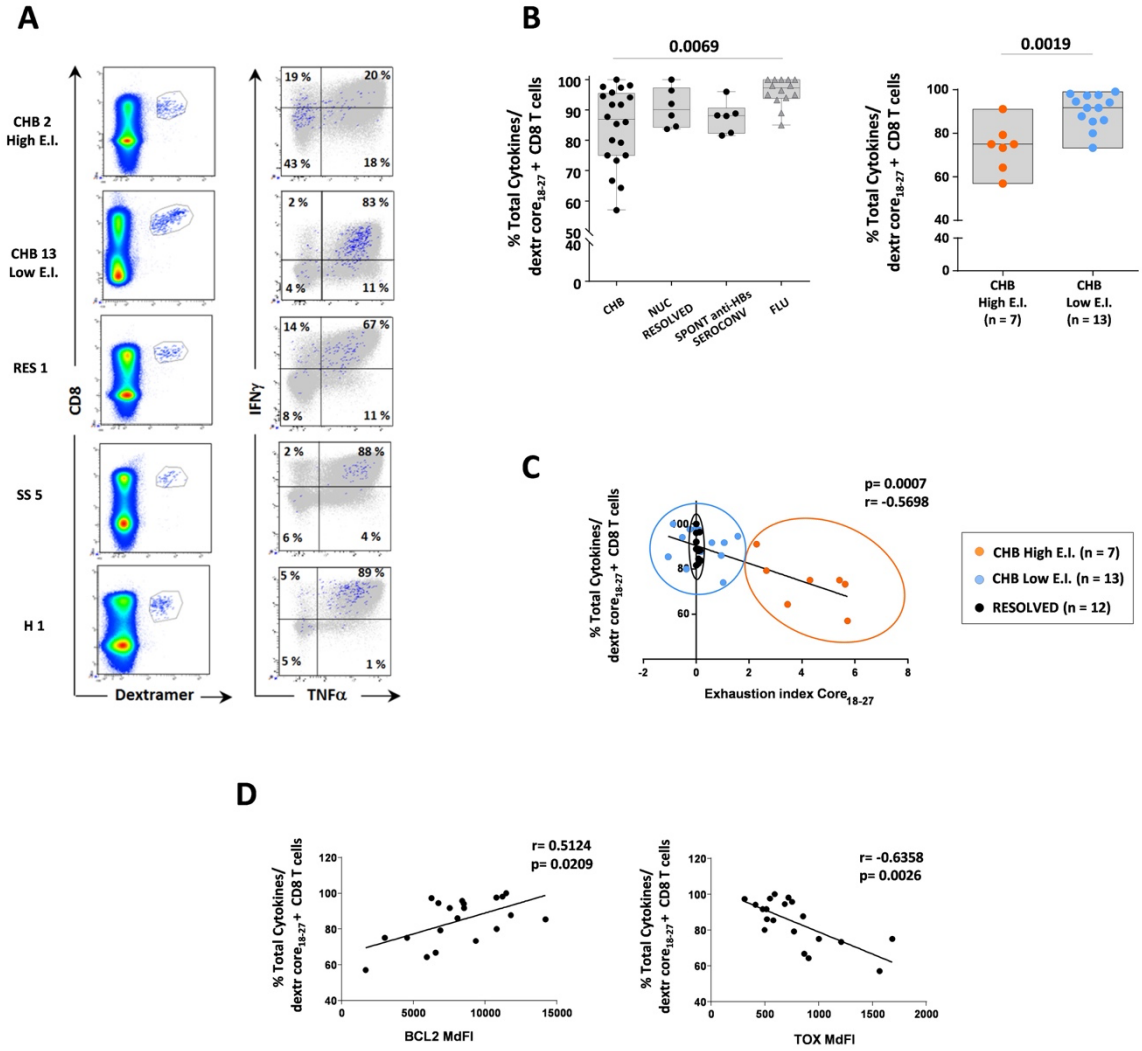

Figure 15. Ex vivo HBV-specific T cell functional analysis. Cytokine production by HBV-specific and influenza-specific CD8 T cells: (A) representative plots; (B) different patient categories assessed after PBMC stimulation with PMA and Ionomycin (box whisker plots, on the left); CHB patients split according to EI values (right graph). (C) Correlation between cytokine production by the core$_{18\text{-}27}$-specific CD8 T cells and EI in chronic patients with high (orange dots) and low (blue dots) EI, respectively, and in HBV resolved patients (black dots). (D) Correlation between cytokine production and Bcl-2 (left graph) or TOX (right graph) expression by core$_{18\text{-}27}$-specific CD8 T cells. Statistics by the Kruskal-Wallis with Dunn's correction test (B) and the Spearman's correlation test (C, D). CHB, chronic hepatitis B; EI, Exhaustion Index; HBV, hepatitis B virus; NUC RES, NUC resolved patients; PBMC, peripheral blood mononuclear cell; SS, spontaneous seroconversion.

CD8 T cell phenotypic profile and response to immune modulatory interventions in vitro

We then assessed whether HBV-specific CD8 T cells of CHB patients with different EI levels, were more or less sensitive to the effect in vitro of antioxidant, polyphenolic and PD-1/PD-L1 targeting compounds[72–78]and a selective toll-like receptor 8 (TLR8) agonist[79,80]. In the absence of these immune modulatory compounds, T-cell lines produced in CHB patients with low EI showed better expansion and cytokine production compared with T cell lines from patients with high EI (Figure 16A, B), in keeping with ex vivo results. All tested compounds had significantly greater effect on HBV-specific CD8 T cells of patients with low EI (Figure 16C–E). Functional changes induced by these immune modulatory compounds are well recapitulated by hierarchical-clustering analysis, which demonstrates a clear segregation of upregulated and downregulated functional parameters in chronic patients with low and high EI (Figure 16E). Interestingly, the anti-PD-L1 monoclonal antibody and the PD-L1 small molecule inhibitor[74] displayed a common clustering indicating similar quality and intensity of CD8 T cell modulation. Likewise, the effects of Resveratrol and the TLR8-agonist clustered together, suggesting a comparable pattern of modulatory activity (Figure 16E). By direct comparison of the different compounds, the TLR8-agonist was the most effective in the reconstitution of the CD8 T cell function.

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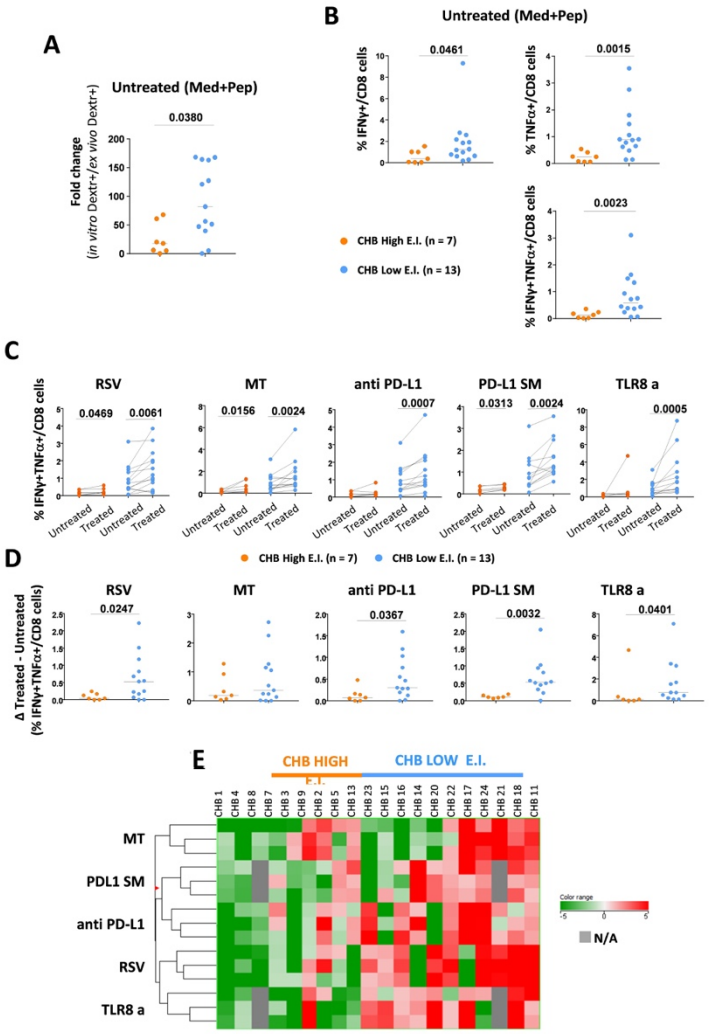

Figure 16. Effect of immune modulatory interventions on the HBV-specific CD8 T cell function. Expansion capacity calculated as the ratio (fold-change) between in vitro and ex vivo frequencies of core$_{18\text{-}27}$ dextramer+ CD8 T cells (A) and percentage of cytokine-positive CD8 T cells (B) in short-term T-cell lines generated by core$_{18\text{-}27}$ peptide stimulation of PBMC from CHB patients with high (n=7) and low (n=13) EI; statistics by Mann-Whitney U test. (C) Percentage of double-positive IFNγ+TNFα+ CD8 T cells in paired short-term T-cell lines generated as in (B) in the presence (treated) or absence (untreated) of Resveratrol (RSV), MitoTempo (MT), anti-PD-L1, a small PD-L1 inhibitor molecule (PD-L1 SM) and a TLR8 agonist (TLR8a) from chronic patients with high (n=7) and low (n=13) EI; statistics by the Wilcoxon-matched-paired test. (D) Delta values of double-positive IFNγ+TNFα+ CD8 T cells derived by subtracting CD8 T cell frequencies of untreated from treated short-term T-cell lines (black lines indicate the median values; statistics by Mann-Whitney U test). (E) Hierarchical-clustering of HBV-specific CD8 T-cell responses induced by in vitro core$_{18\text{-}27}$ stimulation in presence of the different treatments in chronic patients with high (n=7 orange) and low (n=13 blue) EI. CHB, chronic hepatitis B; EI, Exhaustion Index; HBV, hepatitis B virus; N/A, not available; PBMC, peripheral blood mononuclear cell.

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Prediction of immune T cell reconstitution by phenotypic T cell analysis of the whole CD8 T cell population

Evidence of CD8 T cell rejuvenation by immune modulatory treatment in vitro and prediction of response to immune modulation are based on the analysis of a single antigenic specificity and may thus be partially relevant with respect to the multispecific T cell response primed in vivo by infection. Interestingly, we noted a significant positive correlation between core18-27-specific and total CD8 T cells relative to the frequency of PD-1highCD127low/- cells (Figure 17A) and to the expression of differentiation/exhaustion markers, such as Bcl-2 and CD39, and transcription factors, such as TCF-1 and TOX (Figure 17B). This evidence prompted us to assess whether the identification of patients most likely to respond to immunomodulatory compounds in vitro can be accomplished also by phenotypic analysis of total CD8 T cells. Remarkably, the same distribution of patients derived from the calculation of the EI in HBV-specific CD8 T cells, was also found by analysing the expression of different combinations of PD-1, CD127, Bcl-2 and TOX on total CD8 T cells (Figure 17C). In particular, the frequency of PD-1highTOXhigh, PD-1highTOXhighBcl-2-, PD-1highTOXhighTCF1- and PD-1highTOXhighCD127- subsets in total CD8 T cells was significantly greater in CHB patients with high EI. These observations indicate that total CD8 T cell phenotyping can reflect HBV-specific CD8 T cell analysis for the identification of patient cohorts with different antiviral function and different in vitro responsiveness to modulatory compounds. This conclusion is further supported by the significant inverse correlation between cytokine production by HBV-specific T cells and the phenotypic profile of total CD8 T cells (Figure 17C, bottom charts). We then attempted to validate the T cell functional restoration effect of the immune modulatory compounds and the predictive value of the CD8 T cell-based phenotypic score in the context of a multispecific CD8 T cell response stimulated with broader peptide panels covering the entire core and polymerase sequences, which obviously reflect more closely the complexity of the immune response induced by infection. The validation cohort was composed of additional 32 newly enrolled treatment naïve CHB patients (24 and 6 HLA-A2 negative and positive, respectively, details in Tables 2 and 3). Levels of cytokine production were assessed upon PBMC stimulation with 15-mer overlapping peptide pools covering HBV core and polymerase antigens in the presence or absence of the different immune modulatory agents. A frequency threshold of 0.4% PD-1highTOXhigh CD8 T cells among the overall CD8 T cell

population, defined by an ROC curve analysis of phenotypic data generated in HLA-A2 positive patients on core18-27-specific and total CD8 T cells and shown to distinguish patients with different levels of EI values, was applied to analyse the new data set. Results confirm that CHB patients with a PD-1highTOXhigh CD8 T cell frequency below 0.4% show a better response to immune modulatory compounds also when CD8 T cells are stimulated with peptide pools corresponding to the overall core and polymerase sequences able to stimulate a multispecific T cell response (Figure 18A). In line with this observation, a statistically significant inverse correlation was observed between cytokine production by core and polymerase stimulated CD8 T cells and PD-1highTOXhigh CD8 T cell subset frequency (Figure 18B). These experiments confirm the results obtained with HBV-specific CD8 T cells of a single specificity and validate in the context of a multispecific CD8 T cell response the feasibility of distinguishing patients more prone to respond in vitro to immune modulation by a simple phenotypic assessment of CD8 exhaustion parameters.

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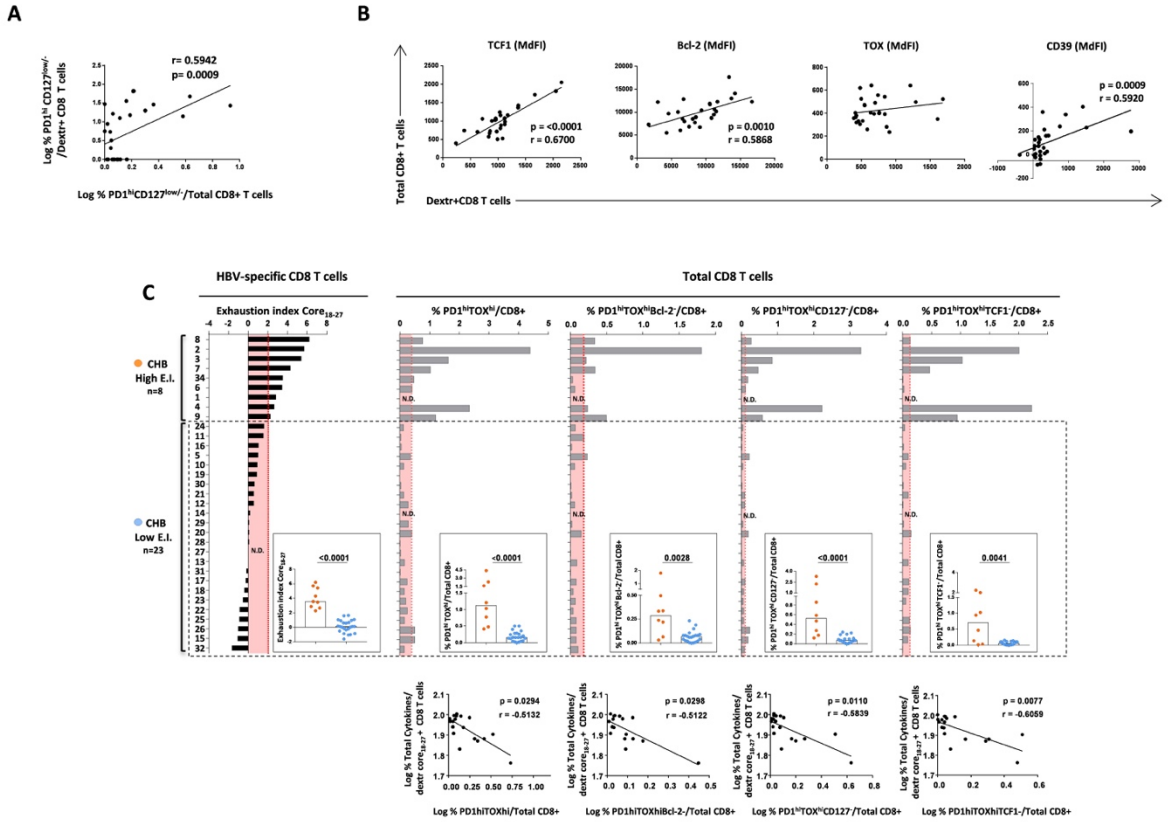

Figure 17. Phenotypic analysis of the total CD8 T cell population. (A) Correlation between logarithmic frequency values of PD-1highCD127low/- T cells among core$_{18\text{-}27}$-specific and total CD8 T cells. (B) Correlations of TCF1, Bcl-2, TOX and CD39 expression in total and HBV-specific CD8 T cells. (C) Top: phenotypic profiles of total CD8 T cells of individual patients (grey bars) in relation to the corresponding EI values derived from HBV-specific CD8 T cell analysis (black bars, on the left). Red areas indicate the significance threshold for each phenotypic CD8 T cell subpopulation calculated by using a ROC curve analysis based on exhaustion index classification criteria (details in online supplemental materials). In the internal squares, each dot represents the frequency of total CD8 T cells expressing the indicated phenotypic profile in chronic patients with high or low EI (Mann-Whitney test). Bottom: correlation between frequency of cytokine positive core18-27-specific CD8 T cells ex vivo and frequency of PD-1high/TOXhigh, PD-1high/TOXhigh/ Bcl-2-, PD-1high/TOXhigh/CD127- or PD-1high/TOXhigh/TCF1- among total CD8 T cells in CHB patients. Statistics by the Spearman's correlation test (A, B, C). CHB, chronic hepatitis B; EI, Exhaustion Index; HBV, hepatitis B virus; ROC, receiver operating characteristic.

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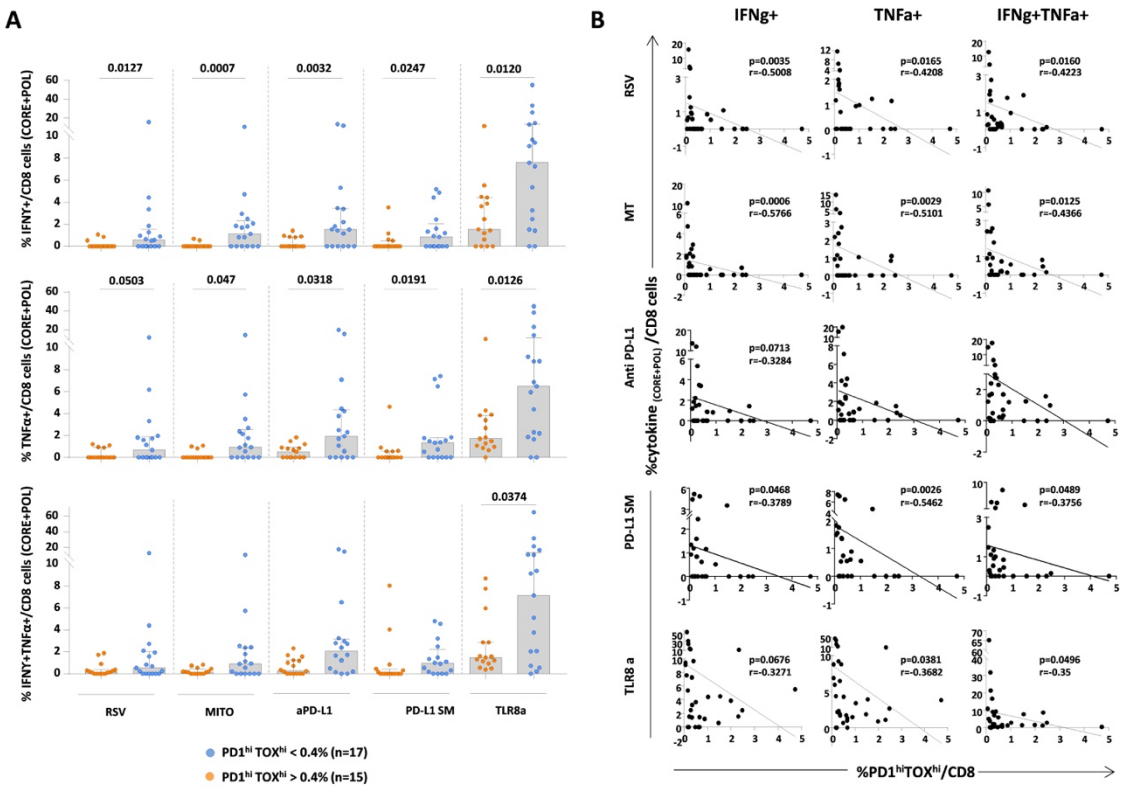

Figure 18. CD8 T cell phenotype can predict recovery of multi-specific T cell responses to immune modulation. Delta values of IFNγ+, TNFα+ and double-positive IFNγ+TNFα+ CD8 T cells derived by subtracting CD8 T cell frequencies of untreated from treated short-term T-cell lines generated by stimulation of PBMC from CHB patients (n=32) with 15-mer overlapping HBV core and polymerase peptide pools. Data are shown as sum of T cell responses against the two antigens. (A) Segregation of individual CD8 T cell responses to core and polymerase peptides based on the PD-1highTOXhigh CD8 T cell frequency threshold (0.4% value) previously obtained using an ROC curve analysis as illustrated in figure 17C. Statistics by the Mann-Whitney U test. (B) Correlation between PD-1highTOXhigh CD8 T cell subset frequency and percentage of IFNγ+, TNFα+ and double-positive IFNγ+TNFα+ CD8 T cells in short-term T-cell lines generated as described above, illustrated for each immune modulation. Statistics by the Spearman's correlation test. CHB, chronic hepatitis B; HBV, hepatitis B virus; PBMC, peripheral blood mononuclear cell.

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DISCUSSION

In chronic HBV infection HBV-specific CD8 T cells appear to be functionally heterogeneous[64,66,69,81,82]. To elucidate further the phenotypic and functional features of this CD8 T cell heterogeneity and to identify CD8 T cell-based predictors of response to immune reconstitution therapies we first selected a homogeneous chronic active hepatitis patient population with persistent viraemia and liver inflammation, eligible for therapy and expected to harbour terminally exhausted HBV-specific CD8 T cells. This is particularly relevant because part of the data so far generated in this area are derived from heterogeneous groups of patients, frequently including also patients with an inactive infection. Particular attention was also paid to identify the rare patient populations able to achieve functional cure of chronic infection either spontaneously or after NUC therapy, which are essential to understand whether reacquisition of a fully protective antiviral activity is achievable after long-term exposure to high antigen and viral loads. Despite low frequency of HBV-specific CD8 T cells, an in-depth phenotypic and functional analysis was performed directly ex vivo.

Second, we studied the effect in vitro of antioxidant, polyphenolic, TLR8 agonist and PD-1/PD-L1 blocking compounds on phenotype and function of HBV-specific CD8 T cells to select optimal candidate targets for immune modulatory therapies. Finally, we searched for predictors of in vitro response to immune modulation, in the perspective of a possible clinical translation aimed at personalising immune therapies for CHB.

By combining the analysis of different exhaustion (PD-1, CD39, TOX) and memory (CD127, Bcl-2, TCF1) markers we identified a specific CD8 T cell phenotypic score, that we named EI, suitable to distinguish cohorts of CHB patients with different CD8 T cell exhaustion levels. By focusing on PD-1 and CD127 staining, we found that a subset of PD-1+CD127+ HBV-specific CD8 T cells was present in all patient categories, irrespective of the clinical condition and level of virus control. This is consistent with the detection of TCF1+CD127+PD-1+ memory like CD8 T cells in chronic HCV infection, that persist after therapy-induced HCV clearance and show higher levels of PD-1 and Eomes expression, as well as reduced cytokine production compared with conventional memory HCV-specific T cells generated after spontaneous resolution of HCV infection[56,83,84].

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Instead, PD-1hiCD127low/- HBV-specific CD8 T cells were present in a more limited proportion of untreated chronic viraemic patients with higher EI levels.

Higher cytokine production was detected in CHB patients with a less exhausted phenotype, marked by low EI levels and the total lack of PD-1hiCD127low/- HBV-specific CD8 T cells. Instead, CHB patients with higher EI levels and a greater representation of PD-1hiCD127low/- CD8 T cells appeared to be significantly less efficient in cytokine production. All together, our observations suggest that the phenotype-based EI score actually reflects the CD8 T cell function and may thus represent a composite multiparametric score for quantification of CD8 T cell exhaustion.

The possibility to rank the level of CD8 T cell exhaustion of single chronic HBV patients may be extremely helpful for the identification of patients with immunological conditions still amenable of functional reconstitution, for example, with antioxidant and polyphenolic compounds, such as MitoQ and MitoTempo or Resveratrol and Oleuropein, that have recently been proposed by us as promising candidates for CD8 T cell-based therapeutic strategies[65,78]. To investigate this issue, we first tested the capacity of different metabolic and immune modulatory compounds to improve HBV-specific CD8 T cell responses in our cohort of chronic viraemic patients with well characterised levels of CD8 T cell exhaustion, and, second, we looked for specific predictive parameters of response to immune modulation. In addition to mitochondrial antioxidant and polyphenolic compounds, HBV-specific CD8 T cells were treated in culture with the TLR8 agonist Selgantolimod and a novel small PD-L1 inhibitor molecule, which represents a promising alternative to monoclonal antibodies[74]. All these compounds induced a significant improvement of CD8 T cell functions, with a slightly better effect of the TLR8 agonist over the other compounds. The effect of the different modulatory agents was greater in low EI patients, namely those with predominance of less exhausted T cell phenotypes.

Although very promising, our results suffer, however, from a major flaw, since our analysis was based on a single epitope specificity. To overcome this drawback, first we tried to select and characterise also polymerase-specific CD8 T cells though they were detected only at very low frequencies and in a limited number of viraemic CHB patients. A simultaneous phenotypic profiling of $pol_{455-463}$- and $core_{18-27}$-specific CD8 T cells was performed in some CHB patients

and, in line with previous studies[69–71], we observed higher levels of TOX and lower levels of PD-1 in $pol_{455-463}$-specific CD8+T cells. These findings highlight intrinsic differences in exhaustion profiles between $pol_{455-463}$ and $core_{18-27}$-specific CD8 T cells in patients with chronic active hepatitis, making the EI score not applicable to $pol_{455-463}$-specific CD8 T cells.

As a second strategy to overcome the single specificity issue, we then investigated whether the subset distribution detected among core-specific CD8 T cells can actually reflect the features of the overall HBV multispecific CD8 T cell population. To do this, we analysed the phenotypic profile of the total CD8 T cell population, assuming that expression of exhaustion markers in the overall CD8 T cell population should be mostly contributed by the multispecific repertoire of the different HBV antigenic specificities. Remarkably, the same stratification of chronic patients with different levels of functional T cell impairement, as defined by the calculation of the EI score for HBV core-specific CD8 T cells, was reproduced by staining total CD8 T cells with PD-1, CD127, TOX and Bcl-2. This means that a simple and easy to be performed phenotypic assessment of the overall CD8 T cell population may allow to predict individual patient's responsiveness to in vitro modulation without the need of virus-specific T cell analysis.

In order to validate the predictive value of the total CD8 T cell phenotypic profile on the response to immune modulation, we then used a different cohort of patients and a different experimental approach looking at the effect of immune modulatory compounds on multispecific CD8 responses induced in vitro by PBMC stimulation with overlapping peptides covering the entire core and polymerase proteins. Results indicate that the phenotypic cut-off value derived from the initial series of experiments with $core_{18-27}$-specific CD8 T cells can be applied to total CD8 T cells from a randomly selected cohort of CHB patients to identify infected hosts who are more or less likely to respond in vitro to immune modulatory compounds. Moreover, these experiments show that CD8 T cell functional recovery by immune modulation is effective also in the context of multispecific T cell responses stimulated by entire HBV proteins. Indeed, functional improvement was observed among both pol-specific and core-specific T cells with a major contribution of the latter to the overall recovery of HBV-specific T-cell responses.

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In conclusion, our study demonstrates that co-expression profiling of specific exhaustion and memory markers by HBV core-specific and total CD8 T cells can allow to generate a predictive score suitable for the identification of distinct CHB patient cohorts with various degrees of CD8 T cell dysfunction and different susceptibility to functional T cell restoration in vitro by immune modulatory treatments. A crucial limitation, however, is that the predictive value of the EI score has been tested only in vitro and that clinical studies have not been performed so far to understand whether this simple phenotypic score can have an application in the clinical setting. This is particularly relevant because without a clinical assessment we cannot know whether the in vitro effect of the tested compounds can be reproduced in vivo and whether the level of restoration detected in vitro can actually translate into a clinically meaningful functional improvement. In favour of our prediction score based on a phenotypic profiling of total CD8 T cells is its simplicity of application which makes it a promising basis for the development of clinically useful T cell-based predictors. Indeed, a clinical trial with mitochondrial antioxidant compounds is being organised in CHB patients to characterise their effect in vivo and to validate the clinical value of our phenotypic CD8 T cell score.

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REFERENCES

1. Liang, T. J. Hepatitis B: The virus and disease. *Hepatology* 49, S13–S21 (2009).
2. Zoulim, F. & Locarnini, S. Hepatitis B Virus Resistance to Nucleos(t)ide Analogues. *Gastroenterology* 137, 1593-1608.e2 (2009).
3. Wei, X. & Peterson, D. L. Expression, Purification, and Characterization of an Active RNase H Domain of the Hepatitis B Viral Polymerase. *Journal of Biological Chemistry* 271, 32617–32622 (1996).
4. Bouchard, M. J. & Schneider, R. J. The Enigmatic X Gene of Hepatitis B Virus. *J Virol* 78, 12725–12734 (2004).
5. Benhenda, S., Cougot, D., Buendia, M.-A. & Neuveut, C. Chapter 4 Hepatitis B Virus X Protein. in 75–109 (2009). doi:10.1016/S0065-230X(09)03004-8.
6. Wei, Y., Neuveut, C., Tiollais, P. & Buendia, M.-A. Molecular biology of the hepatitis B virus and role of the X gene. *Pathologie Biologie* 58, 267–272 (2010).
7. Beck, J. Hepatitis B virus replication. *World J Gastroenterol* 13, 48 (2007).
8. Sunbul, M. Hepatitis B virus genotypes: Global distribution and clinical importance. *World J Gastroenterol* 20, 5427 (2014).
9. Sagnelli, E. Epidemiology of acute and chronic hepatitis B and delta over the last 5 decades in Italy. *World J Gastroenterol* 20, 7635 (2014).
10. Huy, T. T. T., Ngoc, T. T. & Abe, K. New Complex Recombinant Genotype of Hepatitis B Virus Identified in Vietnam. *J Virol* 82, 5657–5663 (2008).
11. Lavanchy, D. Worldwide epidemiology of HBV infection, disease burden, and vaccine prevention. *Journal of Clinical Virology* 34, S1–S3 (2005).
12. EASL Clinical Practice Guidelines: Management of chronic hepatitis B virus infection. *J Hepatol* 57, 167–185 (2012).
13. Jeng, W.-J., Papatheodoridis, G. V & Lok, A. S. F. Hepatitis B. *The Lancet* 401, 1039–1052 (2023).
14. Stroffolini, T. *et al.* The impact of the hepatitis B mass immunisation campaign on the incidence and risk factors of acute hepatitis B in Italy. *J Hepatol* 33, 980–985 (2000).
15. Liaw, Y.-F., Brunetto, M. R. & Hadziyannis, S. The Natural History of Chronic HBV Infection and Geographical Differences. *Antivir Ther* 15, 25–33 (2010).
16. Lampertico, P. *et al.* EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection. *J Hepatol* 67, 370–398 (2017).
17. Busch, K. & Thimme, R. Natural history of chronic hepatitis B virus infection. *Med Microbiol Immunol* 204, 5–10 (2015).

18. McMahon, B. J. The natural history of chronic hepatitis B virus infection. *Hepatology* 49, S45–S55 (2009).

19. Rapicetta, M., Ferrari, C. & Levrero, M. Viral determinants and host immune responses in the pathogenesis of HBV infection. *J Med Virol* 67, 454–457 (2002).

20. E Pato Lo G Y, W. H. *A Clinical Textbook*. www.hepatologytextbook.com (2020).

21. Lau, D. T.-Y. & Bleibel, W. Review: Current status of antiviral therapy for hepatitis B. *Therap Adv Gastroenterol* 1, 61–75 (2008).

22. Zoulim, F., Lebossé, F. & Levrero, M. Current treatments for chronic hepatitis B virus infections. *Curr Opin Virol* 18, 109–116 (2016).

23. Lok, A. S. F. & McMahon, B. J. Chronic hepatitis B. *Hepatology* 45, 507–539 (2007).

24. Arends, J. E., Lieveld, F. I., Ahmad, S. & Ustianowski, A. New Viral and Immunological Targets for Hepatitis B Treatment and Cure: A Review. *Infect Dis Ther* 6, 461–476 (2017).

25. Golsaz-Shirazi, F. & Shokri, F. Hepatitis B Immunopathogenesis and Immunotherapy. *Immunotherapy* 8, 461–477 (2016).

26. Lok, A. S. F. Toward a Functional Cure for Hepatitis B. *Gut Liver* 18, 593–601 (2024).

27. Bertoletti, A. & Bert, N. Le. Immunotherapy for Chronic Hepatitis B Virus Infection. *Gut Liver* 12, 497–507 (2018).

28. Ward, H., Tang, L., Poonia, B. & Kottilil, S. Treatment of Hepatitis B Virus: An Update. *Future Microbiol* 11, 1581–1597 (2016).

29. Marshall, J. S., Warrington, R., Watson, W. & Kim, H. L. An introduction to immunology and immunopathology. *Allergy, Asthma & Clinical Immunology* 14, 49 (2018).

30. Wu, J., Han, M., Li, J., Yang, X. & Yang, D. Immunopathogenesis of HBV Infection. in 71–107 (2020). doi:10.1007/978-981-13-9151-4_4.

31. Chang, J. J. & Lewin, S. R. Immunopathogenesis of hepatitis B virus infection. *Immunol Cell Biol* 85, 16–23 (2007).

32. Guidotti, L. G. & Chisari, F. V. IMMUNOBIOLOGY AND PATHOGENESIS OF VIRAL HEPATITIS. *Annual Review of Pathology: Mechanisms of Disease* 1, 23–61 (2006).

33. Ferrari, C., Missale, G., Boni, C. & Urbani, S. Immunopathogenesis of hepatitis B. *J Hepatol* 39, 36–42 (2003).

34. Rehermann, B. & Nascimbeni, M. Immunology of hepatitis B virus and hepatitis C virus infection. *Nat Rev Immunol* 5, 215–229 (2005).

35. Harty, J. T. & Badovinac, V. P. Shaping and reshaping CD8+ T-cell memory. *Nat Rev Immunol* 8, 107–119 (2008).

36. Chisari, F. V., Isogawa, M. & Wieland., S. F. Pathogenesis of hepatitis B virus infection. *Pathologie Biologie* 58, 258–266 (2010).

37. Kahan, S. M., Wherry, E. J. & Zajac, A. J. T cell exhaustion during persistent viral infections. *Virology* 479–480, 180–193 (2015).

38. Said, Z. N. A. Induced immunity against hepatitis B virus. *World J Hepatol* 7, 1660 (2015).

39. Ganem, D. & Prince, A. M. Hepatitis B Virus Infection — Natural History and Clinical Consequences. *New England Journal of Medicine* 350, 1118–1129 (2004).

40. Wang, L. Crosstalk between innate and adaptive immunity in hepatitis B virus infection. *World J Hepatol* 7, 2980 (2015).

41. Shimizu, Y. T cell immunopathogenesis and immunotherapeutic strategies for chronic hepatitis B virus infection. *World J Gastroenterol* 18, 2443 (2012).

42. Wherry, E. J. & Ahmed, R. Memory CD8 T-Cell Differentiation during Viral Infection. *J Virol* 78, 5535–5545 (2004).

43. Bertoletti, A. Kinetics of the immune response during HBV and HCV infection. *Hepatology* 38, 4–13 (2003).

44. Wherry, E. J. & Kurachi, M. Molecular and cellular insights into T cell exhaustion. *Nat Rev Immunol* 15, 486–499 (2015).

45. Zajac, A. J. *et al.* Viral Immune Evasion Due to Persistence of Activated T Cells Without Effector Function. *J Exp Med* 188, 2205–2213 (1998).

46. Wherry, E. J. T cell exhaustion. *Nat Immunol* 12, 492–499 (2011).

47. Ferrari, C. <scp>HBV</scp> and the immune response. *Liver International* 35, 121–128 (2015).

48. Pauken, K. E. & Wherry, E. J. Overcoming T cell exhaustion in infection and cancer. *Trends Immunol* 36, 265–276 (2015).

49. McLane, L. M., Abdel-Hakeem, M. S. & Wherry, E. J. CD8 T Cell Exhaustion During Chronic Viral Infection and Cancer. *Annu Rev Immunol* 37, 457–495 (2019).

50. Sen, D. R. *et al.* The epigenetic landscape of T cell exhaustion. *Science (1979)* 354, 1165–1169 (2016).

51. Pauken, K. E. *et al.* Epigenetic stability of exhausted T cells limits durability of reinvigoration by PD-1 blockade. *Science (1979)* 354, 1160–1165 (2016).

52. Aranda, F. *et al.* Trial Watch. *Oncoimmunology* 3, e27297 (2014).

53. Paley, M. A. *et al.* Progenitor and Terminal Subsets of CD8 $^{+}$ T Cells Cooperate to Contain Chronic Viral Infection. *Science (1979)* 338, 1220–1225 (2012).

54. Utzschneider, D. T. *et al.* T Cell Factor 1-Expressing Memory-like CD8+ T Cells Sustain the Immune Response to Chronic Viral Infections. *Immunity* 45, 415–427 (2016).

55. Shin, H. *et al.* A Role for the Transcriptional Repressor Blimp-1 in CD8+ T Cell Exhaustion during Chronic Viral Infection. *Immunity* 31, 309–320 (2009).

56. Wieland, D. *et al.* TCF1+ hepatitis C virus-specific CD8+ T cells are maintained after cessation of chronic antigen stimulation. *Nat Commun* 8, 15050 (2017).

57. Kurktschiev, P. D. *et al.* Dysfunctional CD8+ T cells in hepatitis B and C are characterized by a lack of antigen-specific T-bet induction. *Journal of Experimental Medicine* 211, 2047–2059 (2014).

58. Mason, W. S. *et al.* HBV DNA Integration and Clonal Hepatocyte Expansion in Chronic Hepatitis B Patients Considered Immune Tolerant. *Gastroenterology* 151, 986-998.e4 (2016).

59. Kao, C. *et al.* Transcription factor T-bet represses expression of the inhibitory receptor PD-1 and sustains virus-specific CD8+ T cell responses during chronic infection. *Nat Immunol* 12, 663–671 (2011).

60. Khan, O. *et al.* TOX transcriptionally and epigenetically programs CD8+ T cell exhaustion. *Nature* 571, 211–218 (2019).

61. Alfei, F. *et al.* TOX reinforces the phenotype and longevity of exhausted T cells in chronic viral infection. *Nature* 571, 265–269 (2019).

62. Hoogeveen, R. C. *et al.* Phenotype and function of HBV-specific T cells is determined by the targeted epitope in addition to the stage of infection. *Gut* 68, 893–904 (2019).

63. Schuch, A. *et al.* Phenotypic and functional differences of HBV core-specific versus HBV polymerase-specific CD8+ T cells in chronically HBV-infected patients with low viral load. *Gut* 68, 905–915 (2019).

64. Fisicaro, P. *et al.* Pathogenetic Mechanisms of T Cell Dysfunction in Chronic HBV Infection and Related Therapeutic Approaches. *Front Immunol* 11, (2020).

65. Fisicaro, P. *et al.* Targeting mitochondrial dysfunction can restore antiviral activity of exhausted HBV-specific CD8 T cells in chronic hepatitis B. *Nat Med* 23, 327–336 (2017).

66. Cheng, Y. *et al.* Multifactorial heterogeneity of virus-specific T cells and association with the progression of human chronic hepatitis B infection. *Sci Immunol* 4, (2019).

67. Rossi, M. *et al.* Phenotypic CD8 T cell profiling in chronic hepatitis B to predict HBV-specific CD8 T cell susceptibility to functional restoration in vitro. *Gut* 72, 2123–2137 (2023).

68. Gupta, P. K. *et al.* CD39 Expression Identifies Terminally Exhausted CD8+ T Cells. *PLoS Pathog* 11, e1005177 (2015).

69. Heim, K. *et al.* TOX defines the degree of CD8+ T cell dysfunction in distinct phases of chronic HBV infection. *Gut* 70, 1550–1560 (2021).

70. Hoogeveen, R. C. *et al.* Phenotype and function of HBV-specific T cells is determined by the targeted epitope in addition to the stage of infection. *Gut* 68, 893–904 (2019).

71. Schuch, A. *et al.* Phenotypic and functional differences of HBV core-specific versus HBV polymerase-specific CD8+ T cells in chronically HBV-infected patients with low viral load. *Gut* 68, 905–915 (2019).

72. Park, J.-J. *et al.* Hepatitis B Virus–Specific and Global T-Cell Dysfunction in Chronic Hepatitis B. *Gastroenterology* 150, 684-695.e5 (2016).

73. Gane, E. *et al.* Anti-PD-1 blockade with nivolumab with and without therapeutic vaccination for virally suppressed chronic hepatitis B: A pilot study. *J Hepatol* 71, 900–907 (2019).

74. Guzik, K. *et al.* Development of the Inhibitors That Target the PD-1/PD-L1 Interaction—A Brief Look at Progress on Small Molecules, Peptides and Macrocycles. *Molecules* 24, 2071 (2019).

75. Fisicaro, P. *et al.* Combined Blockade of Programmed Death-1 and Activation of CD137 Increase Responses of Human Liver T Cells Against HBV, But Not HCV. *Gastroenterology* 143, 1576-1585.e4 (2012).

76. Boni, C. *et al.* Characterization of Hepatitis B Virus (HBV)-Specific T-Cell Dysfunction in Chronic HBV Infection. *J Virol* 81, 4215–4225 (2007).

77. Kosinska, A. D., Liu, J., Lu, M. & Roggendorf, M. Therapeutic vaccination and immunomodulation in the treatment of chronic hepatitis B: preclinical studies in the woodchuck. *Med Microbiol Immunol* 204, 103–114 (2015).

78. Acerbi, G. *et al.* Functional reconstitution of HBV-specific CD8 T cells by in vitro polyphenol treatment in chronic hepatitis B. *J Hepatol* 74, 783–793 (2021).

79. Daffis, S. *et al.* Toll-Like Receptor 8 Agonist GS-9688 Induces Sustained Efficacy in the Woodchuck Model of Chronic Hepatitis B. *Hepatology* 73, 53–67 (2021).

80. Amin, O. E. *et al.* Therapeutic Potential of TLR8 Agonist GS-9688 (Selgantolimod) in Chronic Hepatitis B: Remodeling of Antiviral and Regulatory Mediators. *Hepatology* 74, 55–71 (2021).

81. Li, Y. *et al.* CXCL13-mediated recruitment of intrahepatic CXCR5+CD8+ T cells favors viral control in chronic HBV infection. *J Hepatol* 72, 420–430 (2020).

82. Heim, K., Neumann-Haefelin, C., Thimme, R. & Hofmann, M. Heterogeneity of HBV-Specific CD8+ T-Cell Failure: Implications for Immunotherapy. *Front Immunol* 10, (2019).

83. Yates, K. B. *et al.* Epigenetic scars of CD8+ T cell exhaustion persist after cure of chronic infection in humans. *Nat Immunol* 22, 1020–1029 (2021).

84. Hensel, N. *et al.* Memory-like HCV-specific CD8+ T cells retain a molecular scar after cure of chronic HCV infection. *Nat Immunol* 22, 229–239 (2021).