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Study of innovative markers associated with hepatitis B virus pathogenesis to guide the development of personalized medical approaches

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List of abbreviations

3TC – *Lamivudine*

ADF – A*defovir dipivoxil*

AF647 – *AlexaFluor 647*

AF700 – *AlexaFluor 700*

AHB – *Acute HBV*

ALT – *Alanine aminotransferase*

APC – A*llophycocyanin*

APC-H7 – A*llophycocyanin-H7*

APCs – *Antigen presenting cells*

AST – *Aspartate aminotransferase*

BFA – *Brefeldin A*

Bim – *Bcl-2-interacting mediator of cell death*

BV – *Brilliant Violet*

cccDNA – C*ovalently closed circular DNA*

CCL-3 – *C-C motif chemokine ligand 3.*

CD – *Cluster of differentiation*

CHB – *Chronic Hepatitis B*

CTLA4 – *Cytotoxic T-lymphocyte-associated protein 4*

DNA – *Deoxyribonucleic acid*

DNAM-1 – *DNAX accessory molecule-1*

DR1 – D*irect repeat 1*

DR2 – *Direct repeat 2*

dslDNA – *Double-stranded linear DNA*

EGFR – E*pidermal growth factor receptors*

EOMES – *Eomesodermin*

ER – *Endoplasmatic reticulum*

ETV – *Entecavir*

FITC – *Fluorescein isothiocyanate*

FVS700 – *Fixable Viability Stain 700*

Gal9 – *Galectin-9*

HBcAg/HBc – *Hepatitis B c Antigen*

HBcrAg – *Hepatitis B core-related Antigen*

HBeAg/HBe – *Hepatitis B e Antigen*

HBsAg/HBs – *Hepatitis B s Antigen*

HBV – *Hepatitis B Virus*

HBxAg/HBx – *Hepatitis B x Antigen*

HC – *Healthy control*

HCC – *Hepatocellular Carcinoma*

HCV – *Hepatitis C virus*

HIV – *Human immunodeficiency virus*

HLA-DR – *Human Leukocyte Antigen – DR*

HSPGs – *Heparin sulphate proteoglycans*

ICIs – *Immune checkpoint inhibitors*

ICS – *intracellular cytokine flow cytometry staining*

IFN – *Interferon*

Ig – *Immunoglobulin*

IL – *Interleukin*

JNK – *c-Jun N-terminal kinase*

LAG-3 – *Lymphocyte-activation gene 3*

LCMV – *Lymphocytic choriomeningitis virus*

LHBs – *Large HBsAg*

mAbs – M*onoclonal antibody*

MACS – *Magnetic activated cell sorting*

MDSC – *Myeloid-derived suppressor cells*

MHBs – *Medium HBsAg*

mRNA – *Messenger ribonucleic acid*

MTC – *Mother to child*

NEAA – *Non-essential ammino-acids*

NK – *Natural killer cells*

NKG2A – *Natural killer group 2 member A protein*

NKG2C – *Natural killer group 2 member C protein*

NKG2D – *Natural killer group 2 member D protein*

NKp46 – *Natural cytotoxicity triggering receptor 1*

NTCP – *Sodium taurocholate co-transporting polypeptide*

NUC – *Nucleos(t)ide analogue*

OLP – *Overlapping peptide pools*

ORFs – *Open reading frames*

PAMPs - *Pathogen-associated molecular patterns*

PBMCs – *Peripheral blood mononuclear cells*

PD-1 – *Programmed cell death protein 1*

PD-L1 – *Programmed Cell Death Ligand 1*

PD-L2 – *Programmed Cell Death Ligand 2*

PE – *Phycoerythrin*

PE-Cy5 – *Phycoerythrin-Cyanine 5*

PE-Cy7 – *Phycoerythrin-Cyanine 7*

Peg-IFN-α – *Pegylated interferon alpha*

Percp-Cy5.5 – *Peridinin chlorophyll protein-Cyanine5.5*

pgRNA – *Pre-genomic RNA*

Pol – *Polymerase*

rcDNA – *Relaxed circular DNA*

rhIL-2 – *Recombinant human interleukin 2*

RIG-I – *Retinoic acid-inducible gene I*

RT – *Reverse transcriptase*

SHBs – *Small HBsAg*

STAT1 – *Signal transducer and activator of transcription 1*

SVPs – *Sub-viral particles*

TAF – *Tenofovir alafenamide*

T-bet – *T-box transcription factor TBX2*

TCF-1 – *T cell factor 1*

TCR – *T cell receptor*

TDF – T*enofovir disoproxil fumarate*

TGF-β – *Transforming growing factor b*

Tim3 – *T cell immunoglobulin and mucin domain-containing protein 3*

TLR – *Tool-Like receptor*

TNF-α – *Tumor Necrosis Factor-alpha*

TOX – *Thymocyte selection-associated HMG-box*

TP – *Terminal protein*

TRAIL – *TNF-related apoptosis-inducing ligand*

TRAIL-R – *TNF-related apoptosis-inducing ligand receptor*

tSNE – *t-Distributed Stochastic Neighbour Embedding*

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1. Sommario

Il virus dell'epatite B (HBV) generalmente provoca un'infezione acuta che si risolve spontaneamente, tuttavia, nei bambini non vaccinati e nei pazienti immunodeficienti, l'infezione può diventare cronica. In questi casi, le cellule natural killer (NK) convenzionali svolgono un ruolo centrale nella mediazione dell'inattivazione dei linfociti T attraverso la sovra regolazione di ligandi inibitori. Uno dei più caratterizzati è il ligando denominato "*Programmed Cell Death Ligand 1*" (PD-L1).

Questo lavoro si concentrerà principalmente sulla caratterizzazione delle cellule T specifiche per l'HBV, delle cellule NK e delle loro interazioni, nonché sull'impatto del PD-L1 in questo meccanismo.

Sono state selezionate due coorti di pazienti affetti da epatite B cronica (CHB). Le cellule derivanti da questi pazienti sono state stimolate con pool di peptidi che coprono la regione del core dell'HBV (OLP) per otto giorni. Le cellule NK sono state separate dalle cellule mononucleate del sangue periferico (PBMCs) tramite isolamento magnetico, ottenendo due popolazioni distinte: PBMC private delle cellule NK e cellule NK isolate. Le PBMC private delle cellule NK sono state stimolate con OLP per sette giorni prima di essere re-stimolate con gli stessi OLP e analizzate tramite citometria a flusso. In alcuni esperimenti le cellule NK sono state attivate con IL-2, IL-12, IL-15, IL-18, trattate o meno con anti-PD-L1 per 1 ora e co-coltivate a rapporti fisiologici (~10% del totale delle PBMC) prima della re-stimolazione.

La funzionalità e la frequenza delle cellule specifiche per l'HBV è stata migliorata significativamente dall'aggiunta delle cellule NK attivate, rispetto alle PBMC stimolate. I pazienti classificati come altamente responsivi, in base al livello di cellule T specifiche per l'HBV in seguito alla stimolazione hanno mostrato una perdita completa o parziale della risposta delle cellule T specifiche per l'HBV in seguito alla deplezione delle cellule NK, mentre i

pazienti scarsamente responsivi non hanno mostrato questo comportamento. Questa perdita viene poi parzialmente ripristinata con l'aggiunta di cellule NK attivate, indipendentemente dal blocco del PD-L1. Inoltre, la produzione di interferone gamma delle cellule NK, in seguito alla stimolazione da parte delle citochine, correla negativamente con il livello di interferone gamma prodotta dalle cellule T CD8 specifiche per l'HBV.

I nostri risultati dimostrano che le cellule T specifiche per l'HBV dei pazienti affetti da CHB mostrano una risposta limitata alla stimolazione antigenica dell'HBV. La deplezione delle cellule NK altera in modo significativo l'attivazione delle cellule T, inoltre, i risultati dimostrano che le cellule NK attivate dalle citochine sono determinanti per potenziare la risposta delle cellule T specifiche per l'HBV. Questo avviene in particolare nei pazienti meno responsivi. Le cellule NK attivate mostrano un aumento di PD-L1 e un fenotipo alterato, più spostato verso l'inibizione. Nel complesso, questi risultati suggeriscono che le cellule NK modulano la funzione dei linfociti T specifici per l'HBV durante l'infezione cronica da HBV e aprono la strada ad ulteriori indagini, per descrivere meglio l'interazione tra le cellule NK e i linfociti T specifici per l'HBV.

2. Abstract

Hepatitis B virus is a non-cytopathic virus that generally leads to a self-resolving acute infection. Despite this, in unvaccinated children, as well as immune deficient patients, the infection become chronic with conventional NK cells playing a central role, mediating the inactivation of T cells through the upregulation of inhibitory ligands. Among these, Programmed Cell Death Ligand 1 (PD-L1) is one of the most well characterized.

Here we will mainly focus on the characterization of HBV-specific T cells, NK cells, and their interactions as well as the impact of PD-L1 on this crosstalk.

Two cohorts of chronic HBV (CHB) patients were cultured and stimulated with HBV core overlapping peptide pools (OLPs) for 8 days in AB media. NK cells were depleted from PBMCs via magnetic cell sorting (MACS). Briefly non-NK cells were labelled with an antibody cocktail and passed through a magnetic column, resulting in two distinct population. The retained cells resulted in the positive fraction, which represents the NK depleted PBMCs, whereas the flowthrough resulted in the negative isolated fraction, which represents the isolated NK cells. NK-depleted PBMCs so obtained were stimulated with OLPs for 7 days prior to restimulation with the same OLPs and flow cytometry staining. In selected experiments NK cells were activated with IL-2, IL-12, IL-15, IL-18, treated or not with anti-PD-L1 for 1 hour and cocultured at physiological ratios (~10% of total PBMCs) prior to the restimulation. Intra cytokine staining were performed to evaluate the expansion of HBV-specific T cells.

Activated NK cells significantly enhanced the functionality and frequency of HBV-specific compared to bulk-stimulated PBMCs. This effect was preserved in the presence of PD-L1 blockade on NK cells. Patients classified as high responders, based on the level of HBV-specific T cells on upon antigenic stimulation, showed a complete or partial loss of HBV-specific T cell response

upon NK depletion, whereas low responders did not display this behaviour. This loss is then partially restored upon addition of activated NK cells regardless of PD-L1 blockade. Moreover, the level of NK activation upon cytokine stimulation negatively correlated with the level of HBV-specific CD8 T cells.

Our findings demonstrate that HBV-specific T cells form CHB patients exhibit limited responsiveness to HBV core OLPs stimulation, sign of a significant exhaustion. While NK cells depletion does significantly alter T cell activation, cytokine activated NK cells has been found to be determinant to boost HBV-specific T cells response, particularly in patients with higher exhaustion level. Activated NK cells exhibit increased PD-L1 and inhibitory yet activated phenotype. Overall, these findings suggests that NK cells modulate HBV-specific T cell function in CHB and paved the way for further investigation, to better depict the crosstalk between NK and HBV-specific T cells.

3. Introduction.

3.1 HBV prevalence

Hepatitis B virus (HBV) is a member of the family of Hepadnaviridae transmitted primarily through direct contact with infected body fluids such as blood. Despite the presence of an effective HBV vaccine, HBV infections are still a global threat with more than 300 million infected individuals and 1 million death each year due to the virus (WHO., 2022). In low-income country and in countries where the vaccine is not mandatory, HBV prevalence is still high, and this poses a risk on the global goal of HBV eradication by 2030 (Ward et al., 2019). In childhood it is typically transmitted from mother to child (MTC) via exposure to infected maternal blood during delivery or perinatally (WHO., 2022). Horizontal transmission has also been observed in children. Despite this, MTC transmission is the principal transmission route for a large proportion of total chronic infections and is dependent on the HBeAg status and HBV DNA load of the mother. HBeAg positivity and maternal viral loads above 10^6 are associated with greater transmission risk (Nelson et al., 2014). In adulthood, intravenous drug use and sexual transmission are prevalent infection routes (WHO., 2022, EASL., 2017). Importantly, transmission before the age of five results in established chronic infection in 90% of cases, whilst only 5% of transmissions in adulthood result in chronic infection (WHO., 2022).

Figure 1 shows all-age prevalence of circulating HBsAg across the world [***Figure 1 – A***] and the all-age death rate for HBV related diseases [***Figure 1 – B***]. The prevalence of HBsAg is more significant in low-income countries and in the Far East region. This is associated with a higher mortality rate in those regions due to HBV-related diseases (GBD., 2022).

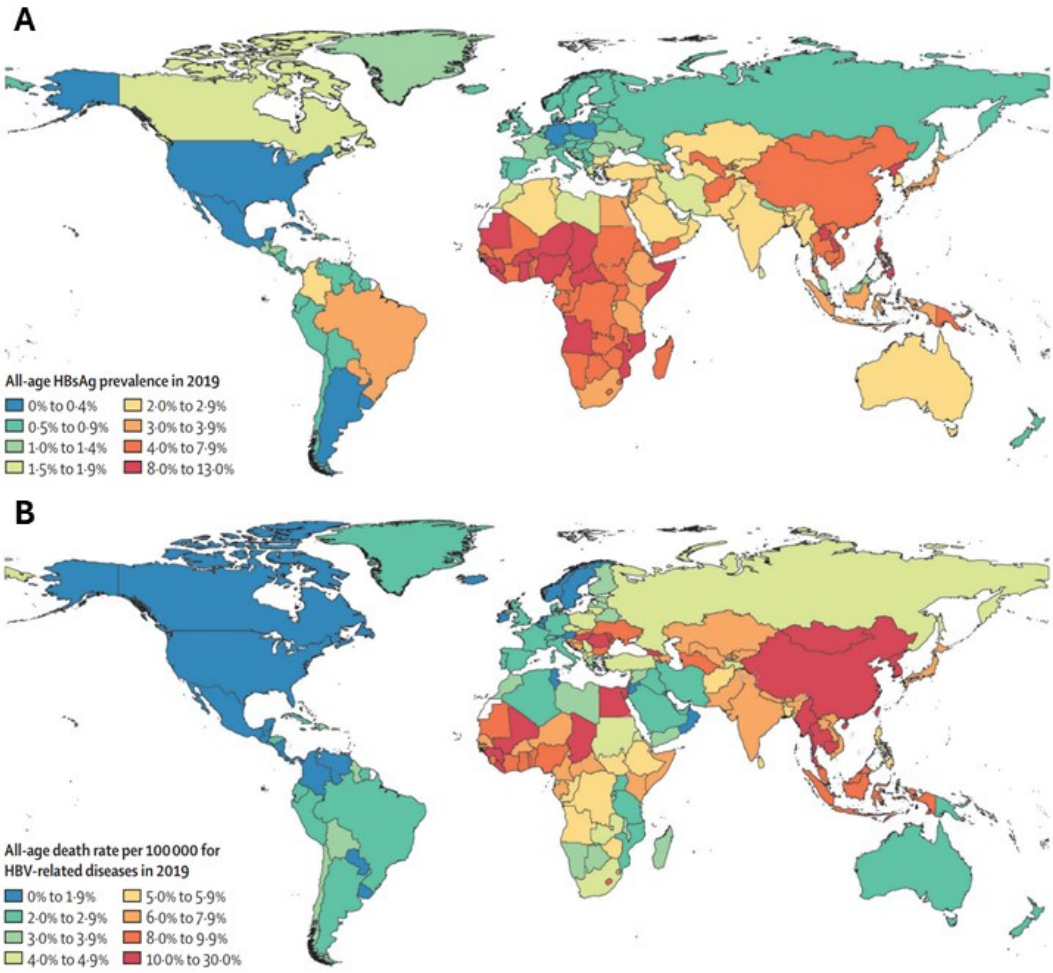

Figure 1. *All-age HBV prevalence (A) and all-age death rate for HBV-related diseases (B).* Image adapted from GBD., 2022.

When HBV infects a healthy individual, it usually generates a self-resolving acute infection. In contrast, individuals who are infected perinatally, in childhood or with immune deficiency conditions, are more susceptible to developing chronic hepatitis (CHB) infection (Nelson et al., 2016, Seto et al., 2018). The chronicity of HBV infection is diagnosed if the patients resulted positive to circulating HBV surface antigens (HBsAg) for more than 6 months from the first diagnosis of HBV infection (Seto et al., 2018, EASL et al., 2017). It is estimated that globally less than 8% of those diagnosed with HBV are receiving treatment (WHO., 2022).

3.2 HBV structure

HBV, also called Dane particle, measures approximately 42nm in diameter and is covered by a host-derived phospholipid bi-layer envelope, embedded with small, medium and large HBsAg proteins [***Figure 2 – A***] (Blumberg et al., 1977, Venkatakrishnan et al., 2016). The inner icosahedral core is composed of the HBV core protein (HBcAg) that surrounds a partially double-stranded genome called relaxed-circular DNA (rcDNA). Viral genome is approximately 3,200 base pairs in length and is covalently bound to the viral DNA polymerase. HBV e antigen (HBeAg) exists as an unbound protein in the space between the inner core and outer envelope [***Figure 2 – A***] (Venkatakrishnan et al., 2016). During HBV replication cycle, the virus can produce several distinct sub-viral particles (SVPs) which may or may not contain HBV nucleic acids. Particles that do not contain nucleic acids are either small spherical and filamentous particles formed of HBsAg or empty enveloped capsids (Dawood et al., 2022, Cossart et al., 1970). SVPs are produced in up to 105-fold greater abundance compared to mature Dane particles [***Figure 2 – B***] (Luckenbaugh et al., 2017, , Blumberg et al., 1977). SVPs that contain HBV nucleic acids are replicative deficient and either contain double-stranded linear DNA (dslDNA) or contain a single stranded pre-genomic RNA (Wang et al., 2016, Delius et al., 1983, Hu et al., 2017).

3.3 HBV Genome and proteome

HBV has a peculiar replication mechanism. Its genomes is one of the smallest among pathogens, organised as rcDNA genome. The genome is transcribed efficiently with multiple AUG initiation sites in four overlapping open reading frames (ORFs), S, C, Pol and X, that encode seven functionally distinct proteins: small (SHBs), medium (MHBs) and large (LHBs) HBsAg components, the X protein, HBeAg, HBcAg, and polymerase [***Figure 2 – C***] (Delius et al., 1983, Dawood et al., 2022). At the 5' end of the negative strand, the terminal protein domain of the viral polymerase is covalently attached with a tyrosyl-

DNA phosphodiester bond, and the 3' end of the positive strand is capped with an RNA primer (Marchetti et al., 2020). The components of HBsAg (SHBs, MHBs and LHBs) are all transcribed from the S-ORF but are under different in-frame promotor control (Marchetti et al., 2020). The preS1 promoter controls LHBs expression, while MHBs and LHBs are controlled by the downstream preS2/S promoter, suggesting there may be separate regulatory mechanisms (Marchetti et al., 2020, Hu et al., 2019) . All S-ORF products have the same carboxy terminus but differ in the length of their amino-terminus (Hu et al., 2019). Common to all HBsAg proteins is the immunodominant α-determinant (aa 124-147). This is the main target for most of anti-HBsAg antibodies (Golsaz-Shirazi et al., 2016). SHBs is the dominant protein produced and constitutes a large proportion of the HBsAg molecules in the viral envelope (Rinker et al., 2020). The LHBs protein is the only protein that carries the PreS1 domain that is key for viral entry into hepatocytes whereases MHBs is highly conserved within Hepadnaviridae, but its role remains unclear, as it is not essential for assembly or infectivity [***Figure 2 – C***] (Yan et al., 2012, Rinker et al., 2020, Ni et al., 2010).

The polymerase protein (pol) is a DNA polymerase with reverse transcription activity. Pol has four distinctly functional domains; the reverse transcriptase (RT), terminal protein (TP) that covalently links to the complete relaxed circular genome, the spacer protein and ribonuclease H (RNaseH) (Lin et al., 2001). Notably, the HBV polymerase lacks a proofreading exonuclease domain [***Figure 2 – C***] (McNaughton et al., 2019). Despite this, HBV mutation rate is lower than in other viruses that rely on reverse transcription such as HIV. Indeed, the partially overlapping structure of its genome limits HBV genetic evolution.

Two functionally distinct proteins, HBcAg and HBeAg, are produced from the C-ORF under the control of the core and pre-core promoters, respectively (Moolla et al., 2002). HBcAg self-assembles to form the icosahedral nucleocapsid. The carboxy-terminal region of HBcAg is found on the inner

nucleocapsid surface and binds nucleic acid to aid reverse transcription of pgRNA and particle maturation. This process if finely tuned by host post-translation modifications apparatus (Tan et al., 2015, Lubyova et al., 2020). The protein product of the precore promoter is the p22 protein which undergoes proteolytic cleavage to form the mature HBeAg and the carboxy-terminal by-product (Slage et al., 2018).

The hepatitis B x protein (HBxAg) is a 154 aa transactivating element required for replication and produced from the X-ORF under regulation by the X-promoter [***Figure 2 – C***] (Slage et al., 2018).

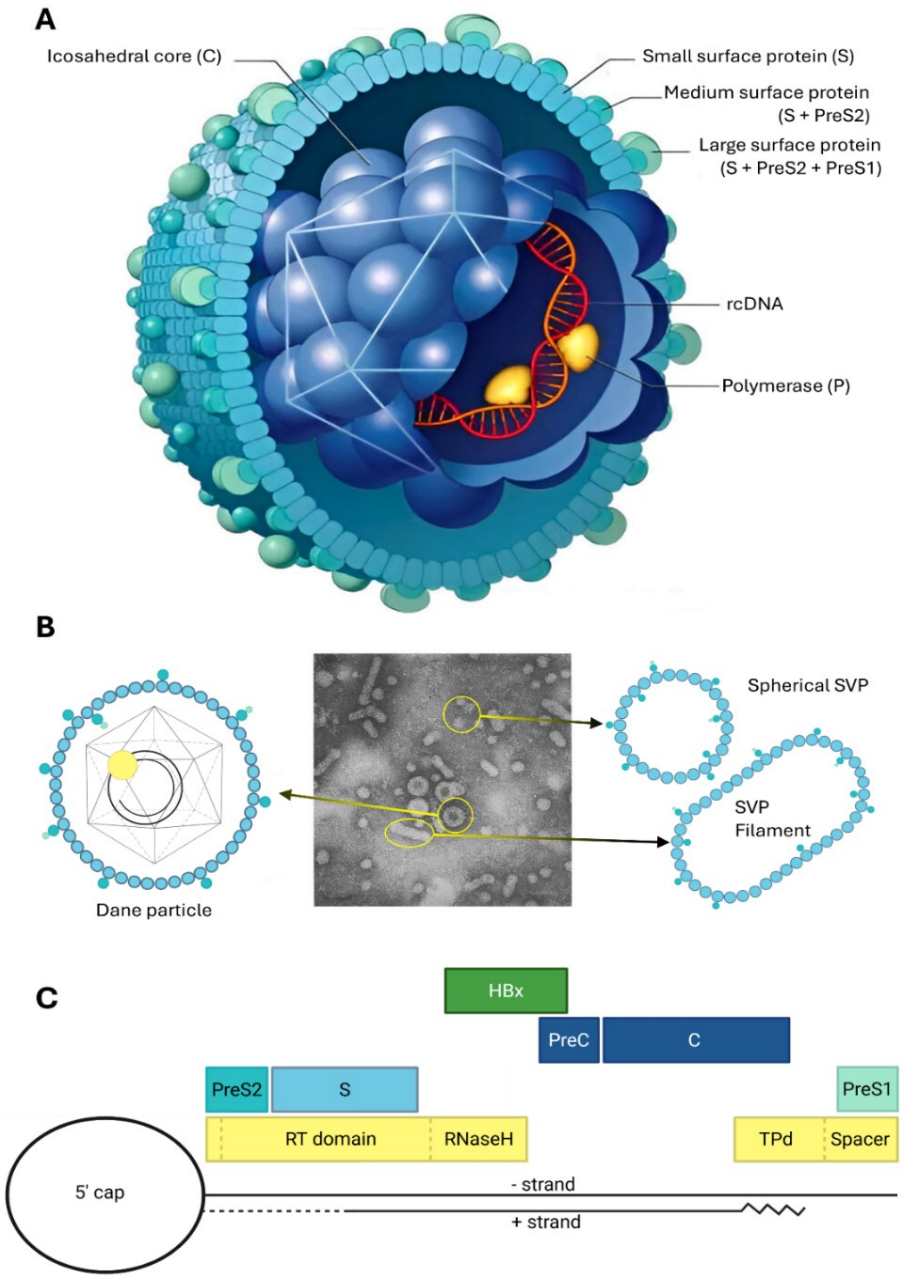

Figure 2. *structure of HBV Dane particle, SVP particles and genome organization*. (A) Structural organization of HBV Dane particle. The inner rcDNA is encapsulated by an icosahedral capsid formed by Core protein. This is surrounded by an envelope of host origin in which small, medium and large proteins are present. Image adapted from Dawood et al., 2022. (B) Tem representation of HBV Dane particle (left) and SVP (either spherical or filamentous – right). Image adapted from Howard et al., 1986. (C) Organization of HBV genome with HBV proteins.

3.4 HBV Replication cycle

HBV has a complex replication cycle with a series of steps unique to the Hepadnaviridae family [***Figure 3***].

HBV replication cycle starts with the binding between preS1 domain of LHBs and heparin sulphate proteoglycans (HSPGs) on the cell surface. This initial binding is not stable and can be easily removed. Thereafter, the amino terminus of the preS1 domain is myristoylated and binds the sodium taurocholate co-transporting polypeptide (NTCP), primarily expressed in the liver. In this process epidermal growth factor receptors (EGFR) act as co-receptors, promoting internalization, even though this mechanism remains unclear [***Figure 3 – Step 1***] (Urban et al., 2009, Iwamoto et al., 2019). The NTCP-bound viral particle is internalised via clathrin-mediated endocytosis, with the viral particle enclosed in an endosome [***Figure 3 – Step 2***] (Herrscher et la., 2020, Harrison et al., 2015). Endosomal pH decrease, is thought to trigger conformational changes in the hydrophobic domain of the LHBs envelop protein, allowing interaction with the endosomal membrane and fusion of the two membranes [***Figure 3 – Step 3***] (Chojnacki et al., 2005). Thus, the nucleocapsid can transits across a network of microtubules, through the cytosol to the nucleus where it interacts with importin-α and β. The genome is then released into the nucleoplasm [***Figure 3 – Step 4***] (Zhao et al., 2021, Kann et al., 2007). Only nucleocapsids containing mature rcDNA genomes are released into the nucleus (Rabe et al., 2003). A key step in HBV replication is the conversion of the rcDNA genome to stable episome-like covalently closed circular DNA (cccDNA). Nuclease and protease dependant mechanisms initiate cccDNA formation by removing the covalently attached pol enzyme from the 5' end of the negative DNA strand. Removal of the pol leaves a residual sequence of redundant DNA that is also removed. The 3’ position of the positive single-stranded DNA acts as a primer for host-derived polymerase, which synthesises the remaining positive strand. The 3’ primer on

the positive strand is removed and completed by host ligase before chromatinization [***Figure 3 – Step 5***] (Zhao et al., 2021, Wei et al., 2021).

Fully repaired cccDNA is the functional template for transcription of the five main mRNA species which is regulated by epigenetic and restriction factors, under the control of the four previously mentioned promoters (core, preS1, preS2 and X) (Hong et al., 2017, Zhang et al., 2014). One crucial regulator is the HBx-protein, which has been implicated in epigenetic transcript activation via histone acetylation and CREB-p300 interactions (Decorsiere et al., 2016). The HBx-protein also activates replication by suppressing restriction factors. The core promoter, which contains the basal core promoter region, is responsible for transcription of both the 3.5kb pre-genomic RNA (pgRNA) and the pre-core mRNA [***Figure 3 – Step 6***]. The core mRNA directs the synthesis of both the HBc subunits and pol once translocated to the cytoplasm, while pre-core transcripts result in the pre-core protein that is post-translationally modified in the endoplasmic reticulum (ER) and processed for secretion. Additionally, activation of the preS1, S2 and S promoters leads to the production of the L, M and SHBs mRNAs, which are subsequently transported to ribosomes on the surface of the rough ER and translated, followed by glycosylation. The resulting proteins are then transported to the cell membrane via the secretory pathway [***Figure 3 – Step 7***] (Hong et al., 2017, Zhao et al., 2021).

There are several alternative forms of viral particles, including those containing double stranded linear DNA (dslDNA). The release of dslDNA into the nucleus results in the formation of a replication incompetent DNA fragment (Yang et al., 1998, Zhao et al., 2021). However, this form of the genome can integrate within the host chromosome, likely through the host repair mechanism of alternative non-homologous end joining (Zhao et al., 2016). Despite integrated HBV genomes cannot produce viral particles, they are a primary source of HBsAg and HBx (Zhao et al., 2016). Additionally, the random integration of the HBV genome may drive the development of HCC as

integration promotes chromosomal instability [***Figure 3 – Step 8***] (Tu et al., 2017).

Assembly of replication-competent viral particles initiates with the pol-protein complexing with the RNA-binding motif at the 5' region (Yao et al., 2019). The formation of the pol-pgRNA complex signals the polymerisation of HBc proteins, resulting in the packaging of the complex [***Figure32 – Step 9***]. Reverse transcription (RT) of the pgRNA into rcDNA occurs in the nucleocapsid containing the ribonucleoprotein complex and the DNA synthesis is initiated. An empty hydroxyl group on a tyrosine motif within pol binds with a dGTP nucleotide, followed by polymerisation of an additional four nucleotides complementary to residues within the 5' region of pgRNA (Yao et al., 2019, Zhao et al., 2021). Pol then folds onto the 3' end of direct repeat 1 (DR1) and synthesises the entire negative strand, while the RNaseH domain within pol degrades the remaining RNA template. A residual RNA sequence containing the DR1 remains after elongating the negative strand and base pairs with an additional direct-repeat region (DR2). This reaction initiates DNA polymerisation and elongation of the positive strand and the formation of the characteristic circular genome. The pol protein remains covalently attached to the rcDNA in the mature viral particle [***Figure 3 – Step 10***]. Capsids with completed HBV rcDNA are then either trafficked to the cell membrane and egress via the multivesicular body secretory pathway into the circulatory system [***Figure 3 – Step 11***] or are recycled back to the nuclear pore complex to amplify the concentration of cccDNA in the nucleus (Kock et al., 2010). The population of viral particles in peripheral blood is heterogeneous. Viral particles containing pgRNA that is either partially reverse transcribed, partially degraded by RNaseH, or in its native form, can be found in the periphery either as naked capsids or enveloped, suggesting these particles egress via a similar pathway to mature Dane particles [***Figure 3 – Step 12***] (Shen et al., 2020, Bai et al., 2018). Virus-like pgRNA particles can be found. This suggest that this species may be a result of viral polymerase failure. Virus-like particles

containing HBV RNA are not able to initiate new infection. However, levels of circulating HBV RNA may reflect the transcriptional activity of cccDNA (Liu et al., 2019).

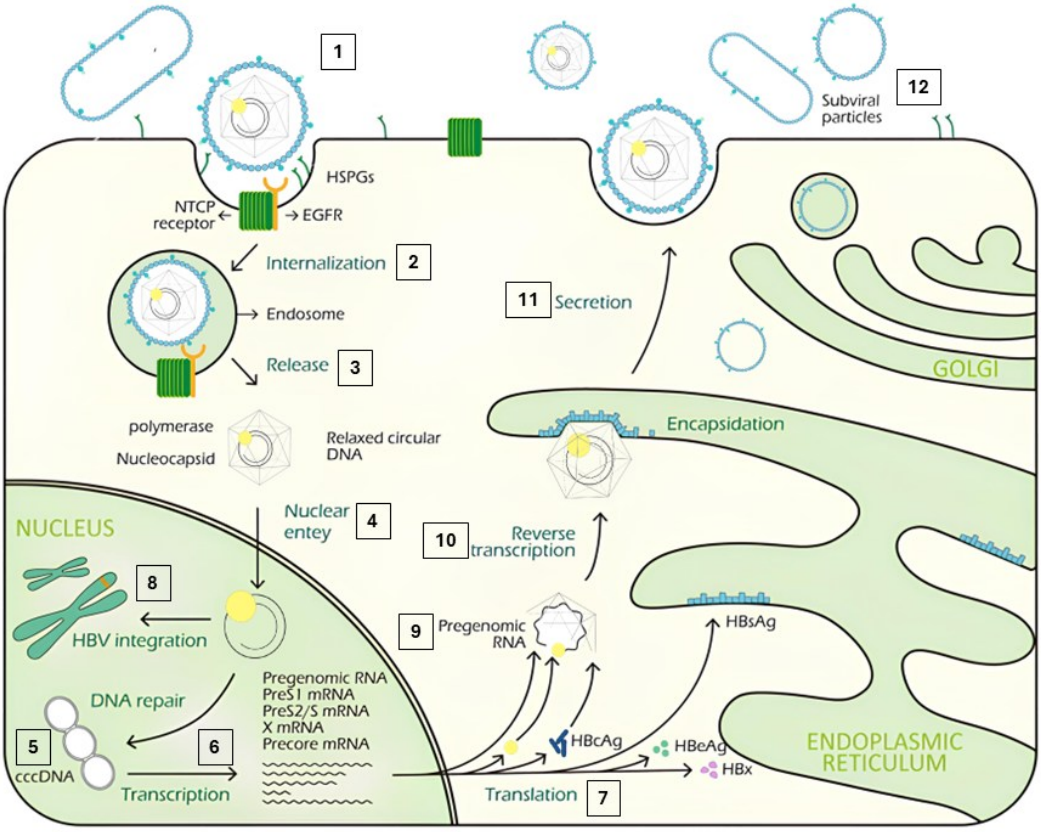

Figure 3. *Schematic representation of HBV life cycle.* HBV infection starts with the recognition of heparan sulphate proteo-glycan on liver surface (1), this promotes the binding with the sodium taurocholate co-transporting polypeptide (NTCP) allowing the viral endocytosis (2). The virus is then release (3) and internalized in the nucleus (4) where it starts its replication, with the formation of the cccDNA (5). This will be transcribed in either mRNA (6) that will be translated in all the viral proteins (7) or pre genomics RNA (9). A fraction of the rcDNA will be integrated in the host chromosome (8) sustaining the production of HBsAg and HBx. Once the pgRNA is transcribed, it is trafficked outside the nucleus where RT occurs (19), followed by encapsidation and secretion of mature viral particles (11) or sub viral particles (12). Image adapted from Zhao et al., 2021.

3.5 Natural history and immune response against HBV

HBV is highly infectious with viral particles that are highly stable and remain infectious for up to 7 days outside the body (EASL et al., 2017). HBV DNA can be detected in the blood approximately 30 to 180 days after transmission and infection results in either self-limiting acute hepatitis or persistence as a chronic infection.

3.6 Acute HBV Infection

Acute hepatitis B (AHB) is characterised by a long asymptomatic period with little evidence of immune activation during this period (Wieland et al., 2004). Once a threshold of replication is reached, an exponential rise in viremia take place. Nevertheless, this process is accompanied by the absence of pro-inflammatory signals, including type 1 and type 2 interferons (IFN) and the enhancement of production of immunosuppressive cytokines, such as IL-10 and lack of adaptive effector function (Dunn et al., 2009). Viral proteins, specifically pol, may actively block the production of IFNs and IFN-stimulating genes by inhibiting Tool-like receptor (TLR) 3, RIG-I and STAT1 (Wange et al., 2010, Chen et al., 2013). Early responses are directed by natural killer (NK) cells, specifically through secretion of IFN-γ, with a consequent decrease in HBV DNA load (Wieland et al., 2004). Eventually, sustained recognition of HBV pathogen-associated molecular patterns (PAMPs) via TLRs results in vigorous innate and adaptive responses (Chen et al., 2013).

Only after partial decrease in viremia, both CD4 and CD8 T cells regain function, become activated and support infection resolution (Dunn et al., 2009). In this process, CD4+ T cells response is fundamental to support both cytotoxic CD8+ T cell and B cell responses. These are required to clear infected hepatocytes and develop lasting protective humoral immunity, respectively. In self-limiting HBV infection, T-lymphocyte responses are typically polyclonal and directed against HBpol and HBx early in infection, while a higher proportion are directed toward HBe, HBs and HBcore following resolution

[***Figure 4***] (Dunn et al., 2009, Bertoletti et al., 2019). During AHB infection, the hepatic damage can manifest clinically with alternated liver functions parameters such as bilirubin and elevations of ALT and AST (Kappus et al., 2013). Almost 20% of AHB individual manifest jaundice (Kappus et al., 2013). An exaggerated immune response during the acute infection can result in acute liver failure or fulminant hepatitis, associated with a 28-day mortality rate of approximately 80% of patients without intervention or transplantation (Wang et al., 2014). In most AHB cases, the cellular and humoral responses are sufficient to develop cellular immunity and neutralising antibodies against HBsAg that are absent during chronic infection [***Figure 4***]. Importantly, cccDNA can persist in the liver of patients with resolved AHBs for decades (Cholongitas et al., 208). Persisting immunity is responsible for lifelong control of HBV and suppression of the immune system, for example during cancer therapy, is associated with a risk of HBV reactivation [***Figure 4***] (Cholongitas et al., 2018).

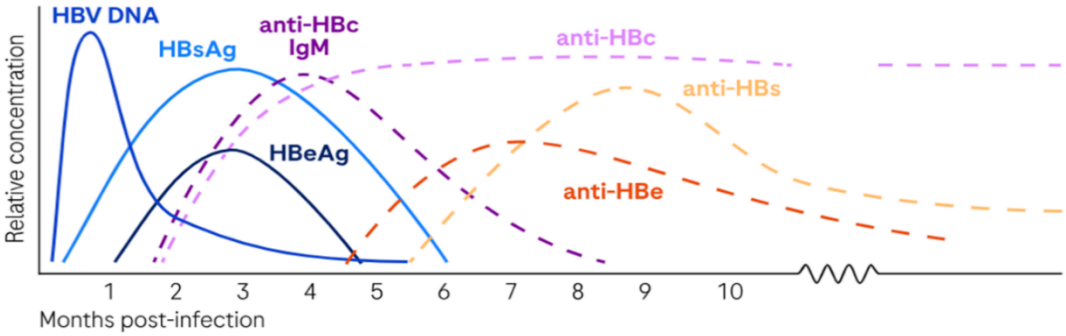

Figure 4. *Clinical manifestation of serum HBV antigen and immunoglobulin during acute HBV infection.* The beginning of AHB starts with a very high viral replication. This is translated with a high burden of HBV DNA detectable in the serum. Following this, some viral antigens can be found at the serum level such as HBsAg and HBeAg, with a peak at around 3-month post infection. After this the level of HBV antigen decreases, with an associated seroconversion and the production of IgM and IgG against HBc, HBe and HBs. In most AHB cases, this is a self-limiting infection. Picture from Roche et al., 2024.

3.7 Chronic HBV Infection

Chronic infection progresses through dynamic stages characterize by HBsAg-positivity, HBeAg status, HBV DNA load and level of liver inflammation, indicated by increased alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels (EASL et al., 2017). Despite being non-cytopathic, the natural history of infection and the resulting pathogenesis of HBV is given by an intricate interplay between the virus and the host immune system. Indeed, chronic HBV infection is characterized by a complex immunological milieu, comprising different immune system cells, from both the innate and adaptive immune system (Bertoletti et al., 2006, Fisicaro et al., 2019, Maini et al., 2013). Among these cell type, it is well established that NK cells and T lymphocytes (both CD4 and CD8) plays a crucial role in the viral clearance and in the development of long-term immunity (Bertoletti et al., 2006, Maini et al., 2013). Indeed, these cell types are the most representative of the liver microenvironment (comprising around 40% the first and 60% the latter). Therefore, the host immune system plays a fundamental role in controlling viral replication and eliminating the viral infected hepatocytes. Despite this, it can fail to control the viral infection, with an exacerbation of immune related cytolysis by NK cell which may leads to liver inflammation and liver damage. Moreover, the constant presence of viral antigens leads to the inactivation of HBV-specific T cells, through a process called exhaustion [**Figure 5**] (Ye et al., 2015, Bertoletti et al., 2006).

The liver is a tolerogenic environment supplied directly with blood from the portal vein(Zheng et al., 2019, Osei-Bordom et al., 2020). Thus, immune cells in the liver are constantly subjected to a high antigenic load. Additionally, due to the network of capillaries, the velocity at which blood travels through the liver is low, resulting in extended antigen exposure. As a result of these factors, the liver has evolved to restrict hyperresponsiveness and contains a high proportion of T-regulatory cells to maintain homeostasis, limiting the potential for runaway and pathological inflammation (Osei-Bordom et al., 2020).

However, this anatomical feature creates an optimal environment for hepatotropic pathogens such as HBV to persist in the presence of a less robust immune response, as compared to other organs.

Chronic infection is defined by HBsAg positivity for more than 6-months after the first HBsAg test and is rarely symptomatic prior to the onset of advanced liver disease (EASL et al., 2017, WHO., 2022).

Anergy and functional exhaustion have been described in many chronic viral infections, including Lymphocytic Choriomeningitis Virus (LCMV), Hepatitis C Virus (HCV) and Human Immunodeficiency Virus (HIV). These infections are characterised by the upregulation of inhibitory markers and a lack of effector function by antigen-activated T cells, NK cells and other mononuclear cells. This process is driven by a continuous T cell activation in an environment with a high antigenic burden (Mueller et al., 2009). HBV produces viral proteins in excessive quantities, which may directly contribute to the characteristic T cell exhaustion observed in people living with chronic HBV infection. The loss of effector function and development of inhibitory markers is progressive. This process involves the loss of homeostatic cytokine recognition and production, increases in inhibitory markers, alterations in the metabolic profile and changes in the epigenetic landscape. Functionally exhausted lymphocytes have a marked decrease in their cytokine mediated responsiveness. The loss of IL-2 response is followed by a production decrease of both TNF-α and IFN-γ and decreased expression of the IL-7 receptor CD127 (Wherry et al., 2003, Bertoletti et al., 2006). Development of antigen-specific TCRs requires a continued supply of IL-7 and IL-15 to maintain the resulting T-memory cells (Wherry et al., 2004). However, a hallmark of exhausted lymphocytes is the loss of IL-7 and IL-15 receptors, thus limiting antigen-independent self-renewal and limiting proliferation (Wherry et al., 2004). Moreover, exhausted lymphocytes have an altered metabolic programme. In a study of *ex vivo* PBMCs derived from people living with chronic HBV infection, it was found that HBV-specific CD8+T cells with exaggerated expression of PD-1 were not able

to use the more efficient mitochondrial oxidative phosphorylation pathway and their energy requirements were instead being obtained with glycolytic pathways [**Figure 5**] (Schurich et al., 2016).

Changes at the transcriptional level also have a profound impact on the development of exhaustion. Factors such as T-bet and TCF-1 are required for the induction of genes for antiviral cytokines, homeostasis and proliferation. All of these are found decreased in chronic HBV infection. Conversely, Eomesodermin (EOMES) has been correlated with the enhancement of production of PD-1, Lag-3 and CD160, all inhibitory receptors and has been found upregulated in CD8 during CHB (Fan et al., 2016, Heim et al., 2020, Wang et al., 2019). Thymocyte selection-associated high mobility group box (TOX) has also been found to be a key transcriptional regulator of T-lymphocyte exhaustion, specifically in chronic HBV infection (Heim et al., 2020). Overall, these factors result in either the reversible non-terminally exhausted T cell incapable of exerting anti-HBV activity or an irreversibly terminally exhausted state. Terminally exhausted cells are also eventually marked for deletion. T-lymphocyte deletion via Fas-FasL, Bim and TRAIL mediated mechanisms have been proposed (Peppa et al., 2013, Allahmoradi et al., 2023). Interestingly, the HBx-protein is implicated in increasing the expression of Fas, suggesting a mechanism of immune evasion operated by the virus (Yoo et al., 2004). Identification of exhausted lymphocyte populations is an intensely researched subject as technologies evolve and allow for expansive marker identification at a single-cell level (Heim et al., 2020, Allahmoradi et al., 2023). However, there is consensus that sustained upregulation of the PD-1 marker partially indicates T cell exhaustion [**Figure 5**].

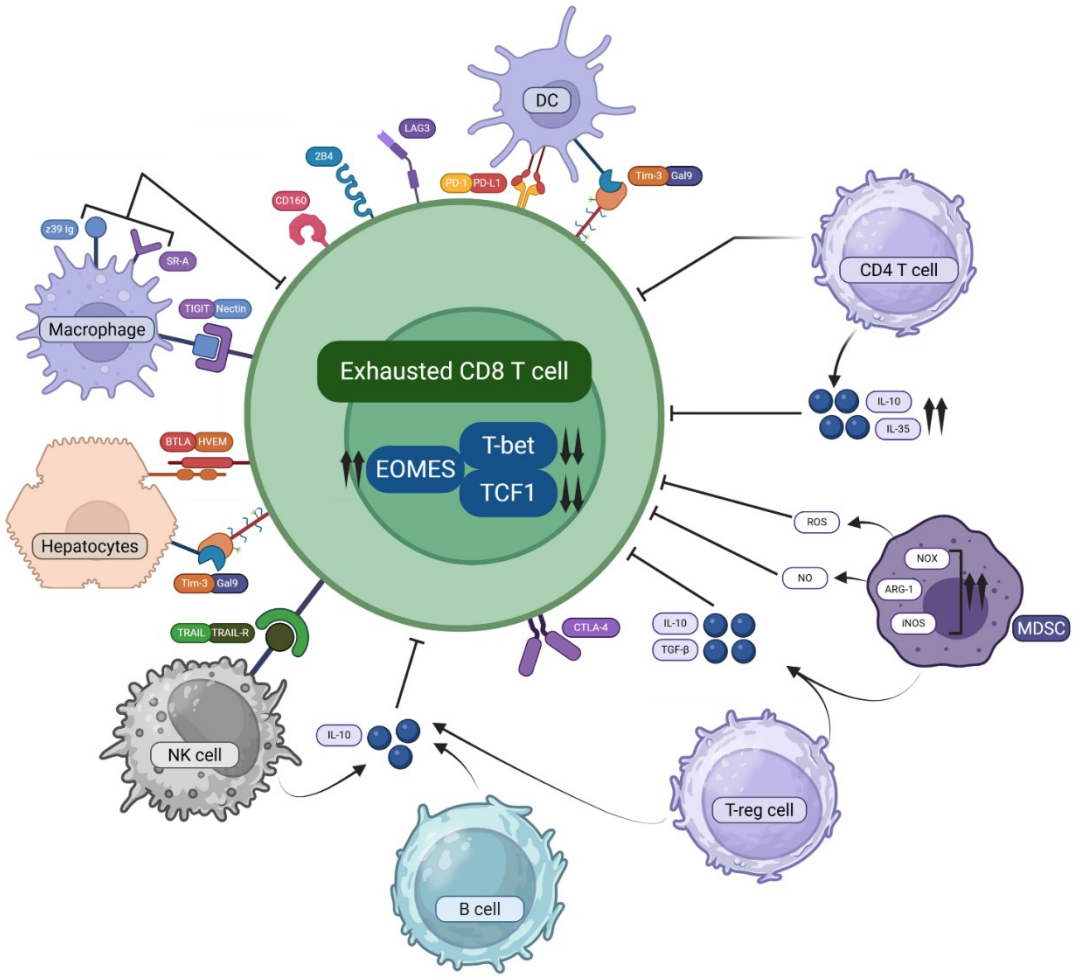

Figure 5. *Representation of immune interaction of exhausted CD8 T cell and other cognate cells during CHB*. The process of CD8 T cell exhaustion is a complex mechanism carried out by hyper stimulated T cell. In this process, the transcriptomic profile of CD8 T cell change, increasing the expression of inhibitory co-stimulatory molecules (e.g. PD-1, LAG3, CD160, CTLA4) and at the same time, decreasing the expression of effector molecule such as IFN-γ and TNF-α. In this process, dendritic cells (DC) promote the inhibition of T cell activation through overexpression of PD-L1 and Gal9. Moreover, CD4, T-reg, B and NK cells, together with myeloid derived stem cells (MDSC) increase the production of inhibitory cytokines such as IL-10, IL-35 and TGF-β, thus promoting CD8 inactivation. Nevertheless, NK cells have been observed increasing the production of TRAIL-R, with the results of CD8 cell death, limiting HBV-specific CD8 expansion by depleting activated T cells. Image adapted from (Allahmoradi et al., 2023)

When T cells are presented with immunogenic protein sequences and become activated, the interaction require a co-stimulatory mechanism such as CD28 expressed on T cells and CD80/CD86 on the presenting cell (Gamez-Diaz et al., 2021). Balance is fundamental in this interaction. The absence of immune checkpoints and negative feedback can be pathological (Gamez-Diaz et al., 2021). The PD-1 pathway is one of the most well-described immune checkpoint pathways. It provides a robust negative feedback signal that may prevent uncontrolled T cell amplification. The ligands for PD-1 are PD-L1 and PD-L2 [**Figure 5**].

The pathway is initiated when a lymphocyte expressing PD-1 binds to a cell expressing PD-L1, which in the context of chronic HBV infection include hepatocytes and non-parenchymal liver cells, as well as intra-hepatic and circulating antigen presenting cells and NK cells (Diniz et al., 2022). In non-chronic infections, PD-1/PD-L1 engagement is transient, and antigen clearance is followed by decreased expression of PD-1. However, in infections with continuous de novo activation events due to a large antigen burden, such as chronic HBV infection, PD-1 and PD-L1 become hyper expressed, and the pathway itself may become a key player in pathogenesis. The PD-1 molecule is encoded by the pdcd1 gene and belongs to the B7 superfamily. PD-1 is a transmembrane protein with an extracellular immunoglobulin-like binding region that interact with PD-L1 on the surface of antigen-presenting cells (Bally et al., 2020). This interaction results in the inhibition of downstream TCR signalling process. This engagement can be overcome in acute infection; however, in chronic HBV infection, the balance is tipped, and PD-1 expression is sustained, as well as TCR inhibition (Schonrich et al., 2019). Downstream consequences of continued PD-1 expression are diverse and include limited expansion capacity, altered metabolism and inability to produce antiviral cytokines such as IFN-γ and TNF-α and the cytolytic molecules perforin and granzyme-B [**Figure 5**].

Broad expression of PD-1 is well defined within the T cell compartment, but this receptor is also expressed by B cells, monocytes, neutrophils and NK cells (Vari et al., 2018, Marotel et al., 2021). During CHB, HBc protein may induce PD-1 on T cells through JNK signalling, thereby aiding immune evasion (Marotel et al., 2021). This is further supported in mouse model studies, where blockade of PD-1 induces IFN-γ production from HBcAg specific T cells (Tzeng et al., 2012). PD-L1 is expressed on a range of cells, such as haematopoietic cells (including T cells and B cells and NK cells), and non-haematopoietic cells such as hepatocytes and non-nucleated cells including platelets. The HBx and HBpol proteins may directly induce over-expression of PD-L1 on hepatocytes via the beta-catenin/c-Myc signalling pathway, limiting the possibility of cytotoxic T cell mediated apoptosis (Sun et al., 2020). The role of PD-L1 in chronic HBV infection outside of the traditional T cell-hepatocyte interaction has recently been evaluated. In a study by Diniz et al. Chronic HBV infection, modelled in mice, not only resulted in upregulation of PD-1 on T-lymphocytes but also led to an increase in PD-L1 on NK cells. NK cells expressing PD-L1 and suppressing CD8+ T cell function could be overcome with PD-L1 blockade, subsequently assisting in boosting CD8+ responses to therapeutic vaccination (Diniz et al., 2022).

3.8 HBV clinical manifestation

Spontaneous clearance of HBsAg and seroconversion to anti-HBsAg is rare, occurring in less than 1% of cases annually in those patients who have previous partial immune control, as evident by anti-HBeAg positivity (Zhou et al., 2019). In patients who sustain lifelong HBsAg seropositivity, there are five main clinical phases that are not necessarily sequential: HBeAg+ infection (previously termed "immunotolerant"), HBeAg+ hepatitis, HBeAg- infection (previously "low-level carriers"), HBeAg- hepatitis, and reactivation [***Figure 6***].

The early phase of chronic infection is characterised by extremely high levels of replication, with high HBV DNA load and HBeAg positivity, but with minimal clinical evidence of liver inflammation in the form of ALT elevations (EASL et al., 2017). During this phase there is a suboptimal virological response to antiviral treatment, for this reason it is not generally recommended (EASL et al., 2017). This phase is the most common outcome for children infected at birth and typically lasts for two or three decades. In this phase, specific HBV immune responses, mostly with Th1 profiles, can be detected but are directed toward a less inflammatory profile with signs of functional exhaustion, including PD-1 expression (Kennedy et al., 2012, Rackaityte et al., 2020). A study conducted by Kennedy et al. on *ex vivo* PBMCs from HBeAg- and immune active chronic HBV display that HBeAg- children and young adults maintain global T cell populations with a greater ability to produce TNF-α but reduced CCL-3 responses, the latter an important chemokine for recruiting innate immune cells. Additionally, they found that PD-1 expression on global populations did not differ from immune active phases, and that younger people with HBeAg+ infection had a greater proportion of HBV-specific responses compared to older patients with immune active chronic HBV. This may partially explain why the use of Peg-IFN-α therapy is partially effective in patients with the HBeAg+ infection disease profile (Zhu et al., 2018).

Although HBV-specific responses are found in this phase, they have a reduced propensity to trigger inflammatory events (Bertoletti et al., 2015). Elevations in serum ALT are not necessarily reflective of the immune-directed pathogenesis or the quantity of specific HBV T cell [***Figure 6***] (Bertoletti et al., 2015). In a study among people with HBeAg+ infection, it was found that hepatocytes were highly expanded and there was evidence of increased HBV DNA integration in the absence of ALT elevations. This suggests that there is low-level immune directed hepatocyte killing occurring among those living with HBeAg+ infection and that mechanisms associated with the development of HCC may already be underway (Masone et al., 2016). Increased age is associated with a tendency of the immune response to shift toward a more inflammatory profile and a transition of patients to subsequent disease profiles and lymphocytes with a greater propensity to activation (Wang et al., 2010).

The HBeAg+ hepatitis phase, also called the immune clearance phase, is characterised by high levels of viral replication and HBV DNA (though generally lower than the HBeAg+ infection profile), HBeAg positivity and elevated ALT levels [***Figure 6***]. It can take decades before this profile emerges for those infected during early childhood (EASL et al., 2017). In a prospective study of 133 children with HBeAg+ infection disease profile, 28% entered the HBeAg+ hepatitis phase at the age of 12-18 years old. As previously mentioned, HBV-specific cytotoxic lymphocytes are already present in the liver during this phase, and the necro inflammatory events associated with ALT elevation are amplified by non-HBV-specific immune responses [***Figure 6***] (Maini et al., 2000). *In vitro* studies have revealed that cytotoxic lymphocytes are likely not the main drivers of hepatocyte damage during HBV clearance. CD8+ T cells were found to efficiently inhibit HBV replication without cytolysis via non-cytolytic viral clearance, through the production of IFN-γ and TNF-α (Philips et al., 2010, Sitia et al., 2004). Changes to the extracellular matrix of hepatocytes by matrix-metalloproteases, induced by cytotoxic T lymphocytes, may lead to

the recruitment of non-antigen specific innate immune cells including NK cells, macrophages, monocytes and dendritic cells. Increased ALT levels are likely a reflection of the activation of these innate immune cells as their absence, even with continued cytotoxic lymphocyte activation, was associated with minimal liver damage in animal models, as previously described (Sitia et al., 2004).

In this process, regulation of hepatocyte damage by negative regulatory pathways such as PD-1/PD-L1 is fundamental, since exacerbation of inflammatory processes can, in rare cases, lead to hepatic decompensation and liver failure (Jeng et al., 2010). Importantly, this active immune environment favours B-cell activation and humoral responses, resulting in the development of anti-HBeAg antibodies and peripheral loss of HBeAg (Vanwolleghem et al., 2015, Fanning et al., 2019). HBeAg seroconversion with development of anti-HBeAg antibodies prior to the age of 30 is beneficial. In contrast, the extension of the HBeAg+ hepatitis phase beyond the age of 40 is associated with the development of liver cirrhosis and HCC (Chen et al., 2010). Following partial control of HBV infection, most patients have a reduction in HBV DNA load and subsidence of hepatic necroinflammation. Antiviral therapy is recommended to be continued in those patients with HBeAg seroconversion prior to attempting discontinuation (EASL et al., 2017). HBeAg- infection is characterised by low levels of HBV DNA, normalised ALT values, HBeAg negativity and minimal necroinflammation or fibrosis [***Figure 6***] (EASL et al., 2017). Moreover, a substantial reduction in both cccDNA activity and pgRNA production can be observed compared to HBeAg+ patients (Suslov et al., 2021). Despite this, there are evidence suggesting that there may be a sub-population within this category that may progress to HCC (Suslov et al., 2021).

Immunologically, HBeAg- patients shows the highest proportion of T-lymphocytes with signs of exhaustion when compared to phases with active disease (Fanning et al., 2019). A study by Boni et al. found that in a prospective

study of 27 HBeAg- patients, with varying levels of viraemia, global T-lymphocyte populations had sustained high levels of PD-1 expression that were inversely correlated with levels of DNA. Despite this, Boni et al found that directly ex-vivo HBV-specific T-lymphocytes were only found in patients with comparatively low levels of HBV DNA and were less likely to produce antiviral cytokines. It is estimated that up to 1.9% of HBeAg- infection patients per year resolve their infection, with the development of anti-HBs antibodies and loss of HBsAg (Invernizzi et al., 2016). The potential for HBsAg loss increases with age (up to ~18% per year after 15 years) and is associated with lower antigenic burden and lower DNA levels early in infection. Spontaneous loss of HBsAg may be predicted by phenotypic analysis of T-lymphocytes. In a study by Xiong et al, the phenotypic characteristics of T-lymphocytes obtained from 141 HBV carriers were analysed. They found that T-lymphocytes from 3 patients who lost HBsAg over the 60-week study period had a distinct immune profile, including upregulation of CTLA-4 and CD107a (Xiong et al., 2021). Identifying true HBeAg- infection status is complex, as it can partially overlap with the clinical presentation of HBeAg- hepatitis, with largely different outcomes. Additional markers such as HBV RNA and HBsAg quantification have been suggested as tools to guide differentiation between HBeAg- infection and hepatitis, where HBV DNA and ALT levels do not provide a clear picture (Liu et al., 2019). In patients with HBeAg- infection, reactivation of cccDNA expression within the nucleus of the hepatocyte may occur spontaneously, through a process yet to be understood, or due to drug mediated immunosuppression with agents such as Rituximab (Loomba et al., 2017).

In a subset of patients who seroconvert to HBeAg-, there is evidence of ongoing transaminitis and HBV replication, though to a lesser extent than patients who are HBeAg+; this is referred to as HBeAg- hepatitis [***Figure 6***] (EASL et al., 2017, Fanning et al., 2019). The infecting HBV of a large proportion of patients in this group harbour mutations in the pre-core region of the C-ORF, specifically G1896A, resulting in a premature stop codon and loss of HBeAg

secretion (Fanning et al., 2019). Additionally, mutations in the basal core promoter sequence, reduce, but do not eliminate, HBeAg production. The pre-core/core region mutations that result in reduced or eliminated HBeAg secretion are likely selected in the HBeAg+ hepatitis phase by immune pressures and may result in stabilisation of pgRNA structure (Wu et al., 2014). Those with HBeAg- hepatitis have higher risk of HCC and progressive fibrosis; thus, antiviral treatment and ongoing surveillance in this population is recommended (EASL et al., 2017, Zeng et al., 2020).

Chronic hepatitis B is a dynamic disease, and not all patients can be described within the categorisation above based on clinical or immunological parameters. In a retrospective study of 4,759 patients with confirmed chronic HBV infection over four years, almost 30% (1,322/4,759) of patients did not fulfil the traditional criteria and were classed as being in a 'Grey Zone" (EASL et al., 2017). Little is known of the outcomes of those within the group, but it has been suggested that they are at increased risk of fibrosis and HCC, while other studies have shown that they may not require therapeutic intervention (Lau et al., 2020, Fanning et al., 2019). The formation of the stable episomal cccDNA within the nuclei of hepatocytes prevents a total cure for HBV. Due to the unique reverse transcription step during replication, targeted therapies are available that reduce HBV DNA load to undetectable levels and partially reduce antigenic load with minimal side effects. However, as cccDNA remains detectable for decades, therapy is often needed for life.

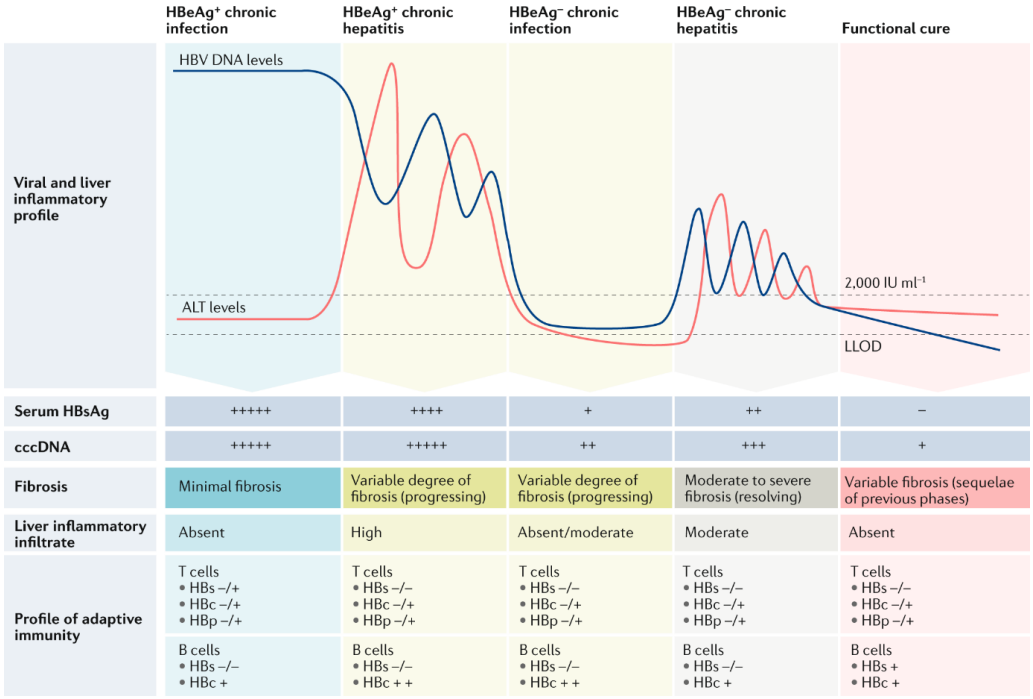

Figure 6. *Virological and immunological characteristics of chronic HBV infection during the four phases of CHB and functional cure.* The HBeAg+ chronic infection is characterized by an elevated level of HBV DNA and HBV replication. This leads to a high production of cccDNA and HBsAg in serum. Despite this the liver inflammation remains absent, with ALT and AST levels undetectable. HBeAg+ chronic hepatitis is characterized by a decrease of viral load level compared to HBeAg+ chronic infection and a decrease of HBsAg production. Despite this decrease, the liver inflammation progresses with variable decrease of fibrosis. This is associated with high ALT and AST and high liver inflammation. HBeAg- chronic infection is characterized by a very low viral replication with HBV DNA and HBsAg barely detectable. ALT ad AST level drastically decreases. Despite this, liver inflammation can still be observed. HBeAg- chronic hepatitis display an increase of virus replication compared to HBeAg- infection with flares of ALT and HBV DNA. This phase is accompanied by a moderate inflammation in the liver. The functional cure phase, achieved by almost 1% of CHB patient per year, is characterized by HBsAg seroconversion, HBV DNA below lowest limit of detection (LLOD) and absent liver inflammation. Image from Fanning et al., 2019.

3.9 Nucleoside or nucleotide analogue (NUC) therapy

NUCs are natural deoxy-nucleo(s/t)ide analogues that are incorporated by the viral polymerase enzyme into DNA, during reverse transcription of pgRNA. However, NUCs lack a 3' hydroxyl group needed to bind the next nucleotide, thus NUC incorporation into a growing DNA strand leads to early chain termination. There are currently five NUCs approved for the treatment of chronic HBV infection; lamivudine (3TC), entecavir (ETV), adefovir dipivoxil (ADF), tenofovir alafenamide (TAF), and tenofovir disoproxil fumarate (TDF) (EASL et al., 2017). Of these, ETV, TAF and TDF are preferred due to high potency and high barrier to the emergence of drug resistance (EASL et al., 2017). The introduction of NUC therapy for people living with chronic HBV infection often leads to the reversal of fibrosis and cirrhosis and reduces the risk of developing HCC even if this therapy does not abolish this risk (EASL et al., 2017, Chen et al., 2006, Udompap et al., 2020). Therapy is only indicated for those with active signs of disease, typically those with HBeAg+ and negative hepatitis (EASL et al., 2017).

NUC therapy is not curative and, in most patients, if therapy is halted, viral replication will immediately restart. Only a proportion of patients who seroconvert to HBeAg- during therapy may discontinue it without virological rebound (Liu et al., 2020). Inhibition of viral replication via RT blockade does not interfere with the production of sub-viral particles containing HBV RNA (Liu et al., 2020). As a result, the ratio of HBV RNA to HBV DNA is modified with the administration of NUC therapy. Excess production of HBV RNA can continue to occur in patients with suppressed HBV DNA (Maasoumy et al., 2020).

There is interest in exploring the direct quantification of HBV RNA in blood as an indicator for cccDNA transcriptional activity. For example, in patients who are virologically suppressed on NUCs, absence of detectable HBV RNA in the serum indicates a reduced risk of viral rebound upon NUC discontinuation (Liu et al., 2020). Additionally, viral genomic sequences integrated into the host

genome are still transcribed, regardless of NUC therapy, although these transcripts mainly consist of HBsAg (Wang et al., 2016). Despite a reduced cccDNA burden in those with HBeAg- infection, there is still a high turnover suggesting there may be long lived sanctuary sites where cccDNA persists (Lythgoe et al., 2021). In a small proportion (~10-12% after 7-8 years of NUCs), immunological control improves to lead to a typically durable loss of HBsAg (EASL et al., 2017).

3.10 Immunomodulators

Therapeutic injection of pegylated interferon alpha (peg-IFN-α) can suppress HBV replication and stimulate immune responses. While the exact mechanism of action of therapeutic interferon remains elusive, it is thought to inhibit several steps of the replication cycle and may stimulate the breakdown of cccDNA (Ye et al., 2021). The overall advantage of peg-IFN-α based therapies is a sustained virological response with a finite treatment duration of one year. However, efficacy is moderate only in few patients and tolerability is poor, with several important contraindications. It is estimated that up to 13-30% of patients achieve a sustained response in optimal conditions (Ye et al., 2021).

Generally, those with low DNA and antigen burden achieve higher response rates with peg-IFN-α therapy alone, but a combination NUC + peg-IFN-α approach may also be effective (Ye et al., 2021).

3.11 Checkpoint blockade

During Chronic HBV pathogenesis, over-activated T cells start to produce inhibitory molecule, inhibiting their antiviral response (Fisicaro et al., 2020). Thus, HBV-specific T cells start to enter in a process of exhaustion with limited proliferation and functional capabilities (Yi et al 2011, Fisicaro et al., 2020). A

prominent field of study to treat CHB focuses on the blockade of inhibitory immune checkpoints, through the use of Immune Checkpoint Inhibitors (ICIs) (Ye et al., 2015, Hoogeveen et al., 2020). ICIs are mostly used in cancer immunotherapies to revert the exhaustion process and have been extensively studied to treat chronic viral diseases such as CHB (Shiravand et al., 2022, Wykes et al., 2018, Casella et al., 2024). Recent studies display a major immune activation after ICI treatment in patients with HCC infected chronically by HBV, compared to uninfected HCC patients, thus indicating an immune microenvironment that is more sensible to ICI in CHB patients (El-Khoueiry et al., 2017, Liu et al., 2023). Different ICI exists with different target proteins. In the context of CHB the most used ones are the PD-1 Inhibitors, PD-L1 Inhibitors, CTLA-4 Inhibitors, with the first two most widely used and well characterized and the latter less used due to its unsafety characteristics (Li et al., 2022, Ye et al., 2015). Moreover, new ICI are being developed such as TIM-3 and LAG-3 inhibitors (Li et al., 2022, Hoogeveen et al., 2020).

ICIs bind to the inhibitory molecule, present on the surface of HBV-specific T cells, competing with the binding of their natural ligand (Hoogeveen et al., 2020). In so doing the inhibitory signalling cascade is interrupted, with the possibility to revert the normal proliferation capabilities of T cell, promoting IFN-γ mediated viral clearance (Fu et al., 2023, Ferrando-Martinez et al., 2021). Recent studies focus on the use of PD-1 and PD-L1 inhibitors, mainly due to the stronger signal given by the molecule(Ferrando-Martinez et al., 2021, Aliabadi et al., 2022, Diniz et al. 2022). Aliabadi et al, through the use of PD-L1 inhibitors display a general increase of IFN-γ and TNF-α *in vitro* production, by HBV-specific CD4 and CD8 T cells from patients with low circulating HBsAg and Hepatitis B core-related antigen (HBcrAg). Moreover, the frequency of pol455-specific T cells increases in patients with low HBcrAg, thus suggesting a more effective role of PD-L1 blockade in patients with lor circulating viral antigens (Aliabadi et al., 2022). It is asserted that, in accordance with this, a high level of functional exhaustion by HBV-specific T cells is associated with

the absence of immune response and this cannot be reverted by PD-L1/PD-1 axis blockade (Ferrando-Martinez et al., 2021). Furthermore, Diniz et al, discover that the blockade of PD-L1 expression on cytokine-activated human NK cells promoted their capacity to enhance the *in vitro* expansion of HBV-specific $CD8^+$ T cells from human donors with CHB, in an interferon-γ–dependent manner (Diniz et al. 2022). These data suggest that PD-L1 blockade may become a therapeutic treatment to improve HBV-specific T cell response and can be used in combination with other ICI or NA to boost the treatment efficacy (Dumolard et al., 2023, Wong et al., 2022).

4. Aim of the study

Hepatitis B virus is a non-cytopathic virus that generally leads to a self-resolving acute infection (Glebe et al., 2013, Yuen et al., 2018). Despite this, in unvaccinated children, as well as immune deficient patients, the infection become chronic due to uncontrolled production of viral antigens (e.g. HBsAg and others) with conventional NK cells playing a central role, enhancing the inactivation of T cells through the upregulation of inhibitory ligands (Yuen et al., 2018, Khanam et al., 2021, Yang et al., 2019). Among them, PD-L1 is one of the most well characterized, interacting with PD-1, expressed on lymphocytes, inhibiting a correct adaptive immune response (Diniz et al., 2022). Studies in mice shows that the blockade of PD-L1 signalling cascade enhance the expansion of HBV-specific T cells and boost the helper function of NK cells, increasing the level of IFN-γ production (Diniz et al., 2022).

The aim of this project is to fully characterize the interactions between NK and CD8 T cells as well as the impact of PD-L1 on this crosstalk.

The project is structured in 3 objectives listed below.

Objective 1: Optimization of the flow cytometry workflow for the analysis of NK cells (phenotypic and functional characterization) and HBV-specific T cells, following stimulation and/or checkpoint blockade. Data obtained in this step will represent the first result: an optimized protocol to be used in the downstream analysis.

Objective 2: Thanks to the experimental approach optimised in objective 1, we could perform an in-depth characterization of NK cells regulation of HBV-specific T cells (with or without stimulation and/or checkpoint blockade) in a cohort of HBV patients recruited within the study. Furthermore, we could correlate the type and magnitude of the regulation with other known clinical, virological or immunological parameters in the available cohort. Based on

these results, we proceed to the study of additional human samples for enrichment of the selected populations.

Objective 3: Assessment of virological and immunological biomarkers in selected cohorts and determination of NK cells regulation of HBV-specific T cells. Phenotypic and functional characterization (e.g. PD-L1, IFN-γ) of isolated NK cells following stimulation and/or checkpoint blockade. Identification of a potential immunological biomarker that can correlate with immune status and potential for reactivation of HBV-specific adaptive immune response.

5. Material and Method:

5.1 PBMCs isolation form CHB patients:

34 patients from 2 different cohort were included in the study. The patients recruited in the study were diagnosed with CHB (HBsAg positivity for more than 1 month). Patients with co-infection (e.g. HIV-1, HCV, HDV) were excluded from the study. Sample sizes were determined by the explorative nature of this study. ***Table 1*** summarizes the clinical characteristics of the patient's sample used in this study. All experiments were carried out in accordance with the principles espoused in the Declaration of Helsinki. The local ethics committee of Verona and Hannover Medical School ensured this project. Written informed consent was obtained from all individuals participating in this study.

Table 1. *Clinical characteristics of patients included in the study.*

	Cohort n°1 (n = 19)	***Cohort n°2 (n = 15)***
Age, Median y (Q1 – Q3) y	42 (37,5 – 50)	42 (35 – 59,5)
Male n° (%)	10 (52,63)	8 (53,33)
Ethnicity, n° (%) Asian Black White	 4 (21,05) 3 (15,79) 12 (63,16)	***N/A***
HBeAg negative, n° (%)	18 (94,74)	13 (86,67)
HBsAg, Median (Q1 – Q3)	631,81 (40,81 – 3573,12)	1163,33 (211,01 – 5345)
HBV DNA positive, n° (%) Median (Q1 – Q3)	11 (57,89) 258 (145,5 – 896)	10 (66,67) 80 (29,5 – 702,5)
ALT, Median (Q1 – Q3)	32 (24 – 34,5)	28 (22,5 – 47)
AST, Median (Q1 – Q3)	19,5 (16 – 23,75)	32 (24,25 – 35,75)
NA therapy at sampling n° (%)	6 (31,58)	15 (100)
Previous IFN therapy n° (%)	1 (5,26)	0 (0)

5.2 PBMCs isolation form CHB patients:

PBMCs were isolated from CHB patients by density gradient centrifugation using Ficoll® following manufacturer's instructions. Cells so obtained were aliquoted and cryopreserved in liquid nitrogen for further use.

5.3 NK isolation from whole PBMCs:

NK cells were depleted from Peripheral blood mononuclear cells (PBMCs) by using Magnetic Activated Cell Sorting (MACS - Miltenyi Biotec) following manufacturer's instructions. Briefly, PBMCs were labelled with a biotinylated antibody cocktail against surface proteins not expressed by NK cells (e.g. CD3, CD4, CD14…). Following the first labelling, streptavidin-conjugated magnetic microbeads were used, thus, non-NK cells were magnetically labelled. Cells so labelled were placed in a magnetic field and both isolated NK and NK-depleted PBMCs were obtained [***Figure 7 – step 1***]. The purity was above 90% and the NK leftover in NK-depleted PBMCs were below 2%.

5.4 Short-term PBMCs culture:

PBMCs and NK-depleted PBMCs were stimulated with 1.25mM core Overlapping Peptides Pool (OLP – Genotype D, subtype ayw) in AB media (RPMI, supplemented with 10% AB serum (human recombinant AB serum), 1% non-essential amino-acids (NEAA), 1% sodium pyruvate, 1% penicillin/streptomycin, 1mM L-Glutamine and 5mM HEPES) for 8 days at 37°C [***Figure 7***]. On day 3 half of the media were replaced with fresh new media containing Human-Recombinant Interleukin 2 (rhIL-2) (20IU/ml) as previously reported [***Figure 7 – step 2***] (Diniz et al., 2022, Aliabadi et al., 2022). On day 8, PBMCs were re-stimulated with 1.25 mM peptide pool, brefeldin A (1 μg/mL) was added 1 hours later, and cells were incubated for 5 hours prior to surface

and intracellular cytokine flow cytometry staining (ICS) [***Figure 7 – step 5, 6***]. In selected experiments, activated NK cells were added to NK-depleted PBMCs at the onset of re-stimulation with/out αPD-L1 treatment [***Figure 7 – step 4, 5***].

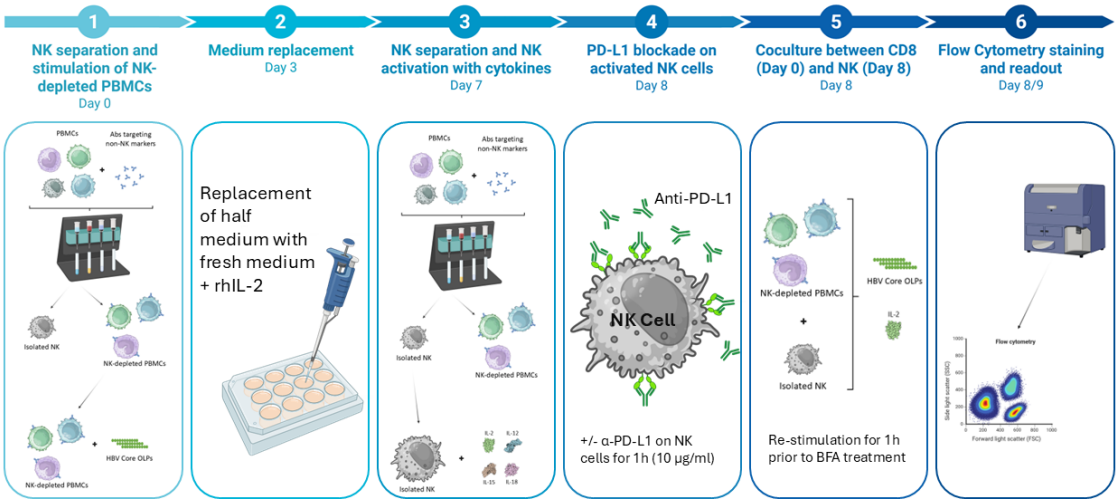

Figure 7. *Schematic workflow for culturing and stimulation of PBMCs.* (1) PBMCs were thawed, and NK cells were removed from PBMCs. Obtained NK-depleted PBMCs were stimulated with Core OLP for 8 days. On day 3 half media were replaced with media containing rhIL-2 (2). In selected experiments, NK cells were activated with cytokine for 24h (3) and treated with αPD-L1 for 1 hour (4) prior to coculture and re-stimulation of conjugate peptide-expanded NK-depleted PBMCs (5). Flow cytometry was performed 6 hours after the onset of re-stimulation (6).

5.5 Cytokine activation of NK cells:

NK cells were isolated as previously described in [2.2] and cultured in AB-media in presence of rhIL-2 (200 IU/ml), IL-12, IL-15, and IL-18 (50ng/ml each) for 24h [***Figure 7 – step 3***]. After activation, NK cells were washed twice and cocultured with stimulated NK-depleted PBMCs. Where reported, activated NK cells were treated with αPD-L1 antibody clone MIH1, 10µg/ml) for one hour, prior to coculture with stimulated NK-depleted PBMCs [***Figure 7 – step 4, 5***] (Diniz et al., 2022).

5.6 Flow cytometry staining and readout:

Multiparametric flow cytometry was used for phenotypic and functional analysis of PBMCs [***Table 2***]. Cells were stained with Fixable Viability Stain 700 (FVS700, BD) before incubation with saturating concentrations of surface mAbs diluted in FACS buffer (PBS supplemented with 1mM EDTA and 2% FCS) for 20 min at 4°C. Cells were fixed and permeabilized for further functional assessment with Foxp3/Transcription Factor Staining Buffer Set (Invitrogen) according to the manufacturer's instructions. For ICS, samples were then incubated with saturated concentrations of intracellular mAbs for 30 min at room temperature, diluted in permeabilization buffer (Invitrogen). All samples were acquired on BD Fortessa X20 flow cytometer (BD Biosciences) and analysed using FlowJo.

Table 2. *Flow cytometry panels used for the analysis of T cells (left) and NK cells (right).*

T cell panel	
Cellular Target	Fluorochrome
HLA-DR	FITC
PD-1	PE
CD3	PE/Dazzle594
Tim3	PE-Cy5
CD8	PE-Cy7
CD4	APC
FVS700	AF700
CD14/CD19	APC-H7
IFN-γ	BV421
CD57	BV510
CD45RA	BV605
CD107a	BV650
CD27	BV711
CD38	BV786

NK cell panel	
Cellular Target	Fluorochrome
DNAM-1	FITC
NKp46	PE
NKG2A	PE/Vio615
CD56	Percp-Cy5.5
NKG2D	PE-Cy7
CD49a	AF647
FVS700	AF700
CD3/CD14/CD19	APC-H7
IFN-γ	BV421
CD57	BV510
NKG2C	BV605
CD107a	BV650
PD-L1	BV711
CD16	BV785

5.7 Data analysis and dimensionality reduction

Flow cytometry data were analysed using FlowJo software (V.9.9.4 and V.10.6.2). Immunophenotype of NK cells, as well as CD4 and CD8 is performed by using the gating strategy represented in ***Figure 8 – A*** for CD8 and CD4 an in ***Figure 8 – B*** for NK cells. Mann-Whitney, Kruskal-Walis and Spearman correlation tests were performed using GraphPad Prism 10 (GraphPad Software) and R V 4.2.2 (2024-11-10, https://www.r-project.org).

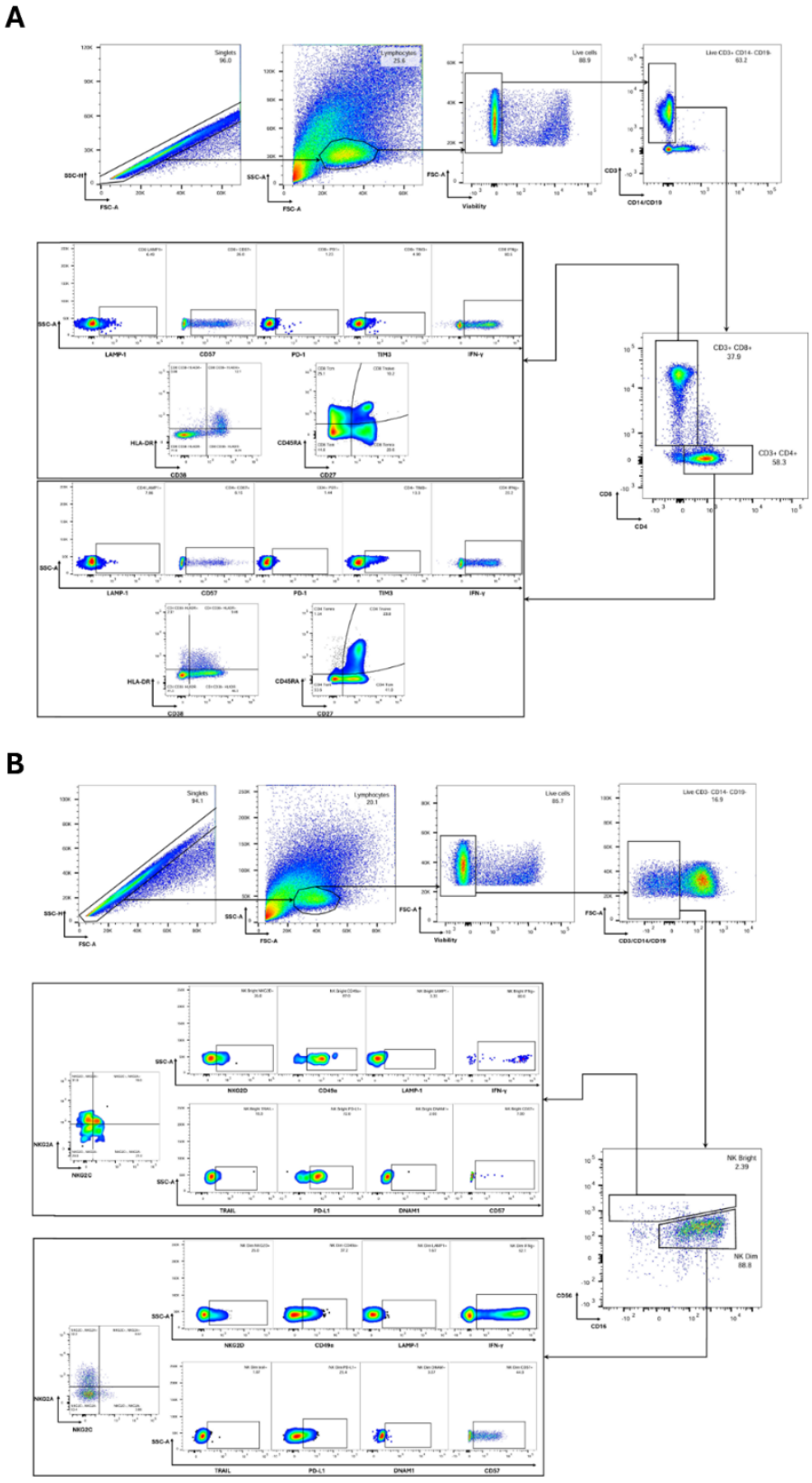

Figure 8. *Schematic representation of CD4 and CD8 (A) and NK (B) gating strategy used for the immunophenotyping of CHB patients.*

For dimensionality reduction, raw NK data were processed with R via FlowAi algorithm to detect anomalies in the flow rate, flow signal and flow margin. ***Figure 9*** shows an example of FlowAi output, along with the clean fcs data. ***Figure 9 – A*** shows the output of flow rate control. The plot reconstructs the flow rate with a resolution of 1/10 of a second. Anomalies in the flow rate are identified with an algorithm based on the generalised ESD outlier detection method. The anomalies are circled in green. ***Figure 9 – B*** shows the anomalies detected in the signal acquisition check. The more stable region selected consistent for all channels is highlighted in yellow. If the removal of outliers has been required before the execution of the changepoint analysis, the detected outliers are circled in green. The FCS file was divided in 409 bins; hence each bin contains 500 event(s). The stable region is located between the bins 1 and 409. ***Figure 9 – C*** shows the dynamic range check results. The plot shows where the anomalies occur the most. The x-axis scale is complementary to the one of the signal acquisition plots. Cleaned flow cytometry data were gated to exclude doublet, dead cells, along with CD3, CD14 and CD19 cells and NK cells were identified based on CD56 expression. Gated NK cells from all the patients were concatenated and tSNE algorithm were used. To identify cluster in the tSNE plot, FlowSOM algorithm were used and events in the plot were clustered in NK Bright and NK Dim based on the expression level of CD56 and CD16.

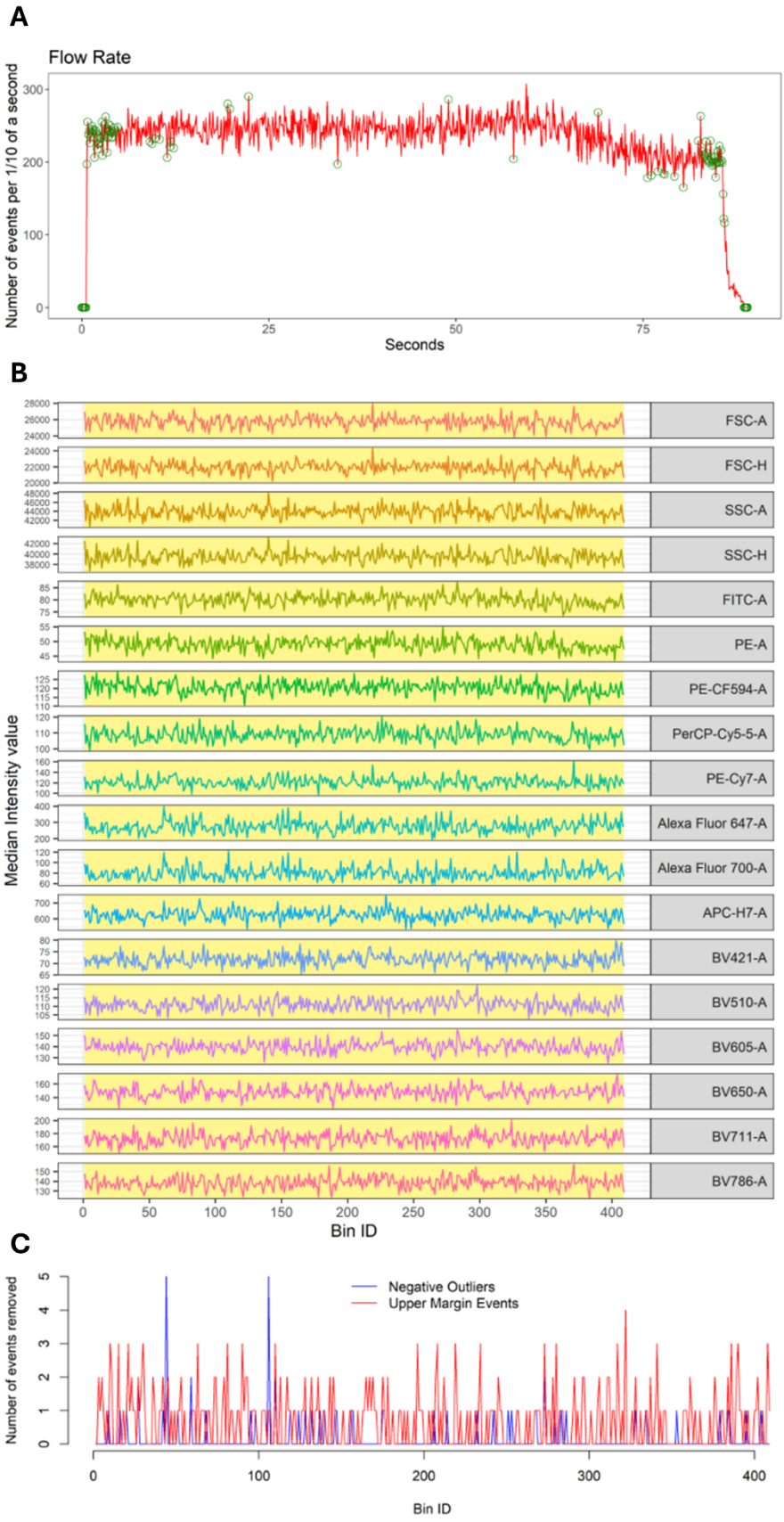

***Figure* 9.** *Output of FlowAi quality check on Flow rate (A), signal acquisition (B) and dynamic rate (C).*

6. Results

***Objective 1**: Optimization of the flow cytometry workflow for the analysis of NK cells (phenotypic and functional characterization) and HBV-specific T cells, following stimulation and/or checkpoint blockade. Data obtained in this step will represent the first result: an optimized protocol to be used in the downstream analysis.*

6.1 NK cells depletion to study the impact of NK in HBV-specific T cells response

We spent a lot of efforts to establish a functional protocol for the study of the relationship of NK and HBV-specific T cells response.

The first step was to optimize the condition to obtain a purified and enriched NK population in order to minimize the contamination of the PBMC/ CD8 cells.

To separate NK cells from PBMCs, both Negative and Positive NK magnetic isolation kits available in commerce were used. A fraction of separated cells was stained and analysed via flow cytometry. The data were analysed using the gating strategy described in [***Figure 10***].

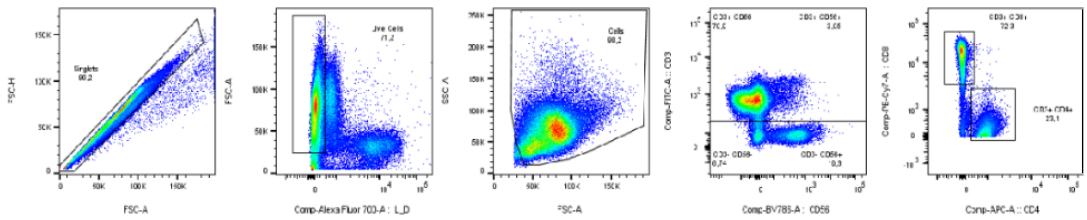

Figure 10: Gating strategy used for the analysis of NK and CD8 purity after the magnetic separation.

After the separation, NK negatively selected showed a better purity compared to the one positively selected with a lower proportion of contaminants expressed as non-NK cells (CD3+ CD8+, CD3+ CD4+, CD3- CD56-) [***Figure 11 – A***]. In detail, NK negatively selected display a 0,15% of CD8 contamination, compared to the 29,09% of CD8 contamination of the positively selected. Despite this, NK-depleted PBMCs display a proportion of NK still present after the separation, independently from the kit used [***Figure 11 – B***].

From now on, only the negative NK isolation kit will be used, thanks to its improved results. NK cell depletion from PBMCs will be subsequently optimized in [**6.2**].

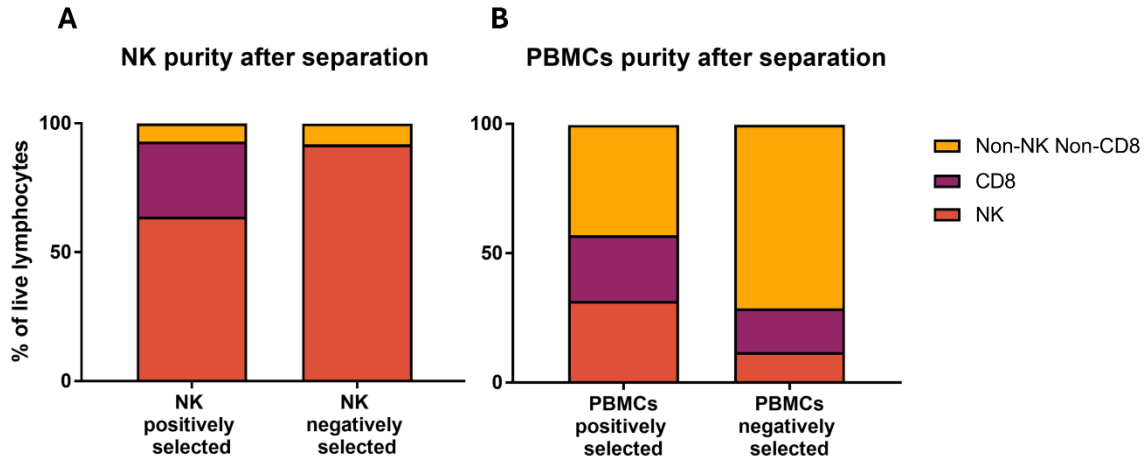

Figure 11: NK (A) and PBMCs (B) purity after the isolation of NK using either the positive or the negative magnetic isolation approaches. NK negatively selected (A – Right column) display a better purity compared to NK positively selected (A – Left column). Furthermore, using both the two approaches, PBMCs display a fraction of NK still present after the isolation (B).

6.2 Optimization of NK cell depletion

NK cells were depleted from Peripheral blood mononuclear cells (PBMCs) by using Magnetic Activated Cell Sorting (MACS - Miltenyi Biotec) following manufacturer's instructions. Briefly, PBMCs were labelled with a biotinylated antibody cocktail against surface proteins not expressed by NK cells (e.g. CD3, CD4, CD14). Following the first labelling, streptavidin-conjugated magnetic microbeads were used, thus, non-NK cells were magnetically labelled. Cells so labelled were placed in a magnetic field and ***isolated NK*** cells were obtained. Subsequently, 3 more washes were performed on ***NK-depleted PBMCs*** in order to reduce the level of contaminants in it, intended as NK cells. After NK isolation, NK-depleted PBMCs display a reduction of NK cells (defined as CD3- CD56+) [***Figure 12 – A, B centre graphs***]. Similarly, isolated NK cells show a very high frequency of NK cells, with very low to non CD4 and CD8 T cells [***Figure 12 – A, B right graphs***]. Overall, the reduction ratios of NK cells in NK-depleted PBMCs were above 80% [***Figure 12 – C***], and the NK purity of isolated NK were above 90% [***Figure 12 – D***].

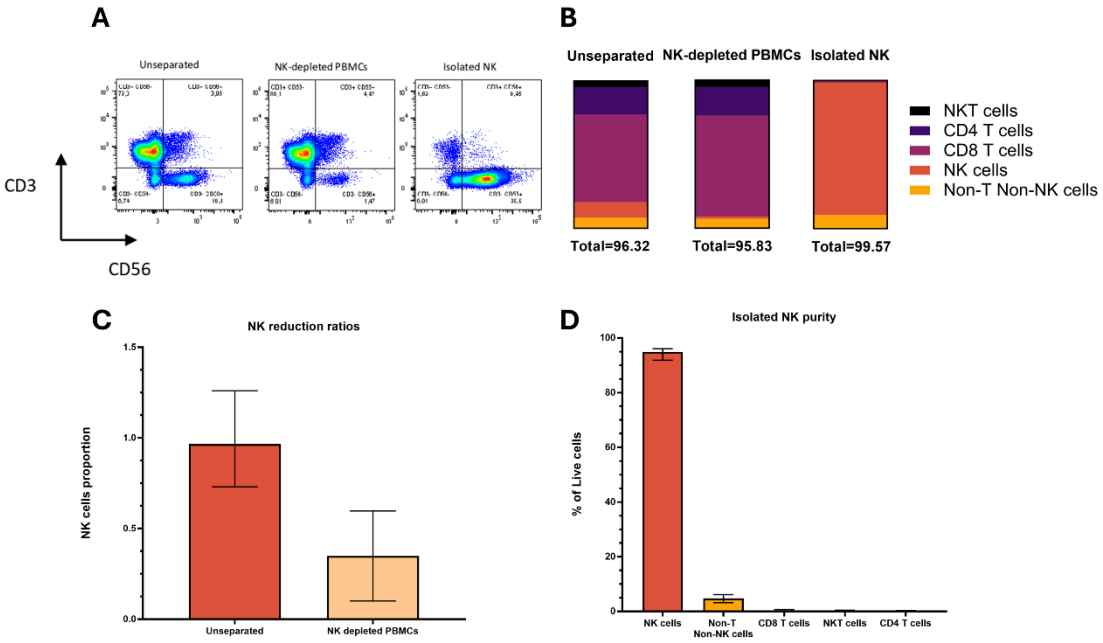

Figure 12. *Purity of NK depleted PBMCs and Isolated NK after NK isolation.* (A) Example of NK cells isolation performances. From left to right, gates represent the proportion between NK cells in whole PBMCs (left), in NK-depleted PBMCs (centre) and in isolated NK (right). (B) NK isolation performances represented by stacked column graphs. NK cells (orange bar) were highly reduced in NK-depleted PBMCs. (C) Relative decrease of NK cells in NK-depleted PBMCs after NK isolation. NK-depleted PBMCs displays a reduction ratio above the 80%. (D) Purity of isolated NK were above 90%, little to no CD4/CD8 detection can be seen.

Objective 2: *Characterization of NK cells regulation of HBV-specific T cells with or without stimulation and/or checkpoint blockade*

6.3 Minimal HBV-specific T cells response in both CD4 and CD8 T cells upon HBV stimulation

PBMCs from Chronic HBV (CHB) patients were thawed and stimulated with HBV core OLP for 8 days in presence of rhIL-2. Cells were re-stimulated at day 8, prior to flow cytometry staining as reported in [**5.5**]. Data so obtained were gated using the gating strategy reported in ***Figure 8*** *and analysed with Mann-Whitney test*. Here we will specifically focus on the intracellular IFN-γ production, which reflect the activation of the HBV-specific T cells (Aliabadi et al., 2022).

As indicated by ***Figure 13 – A***, the immune response to HBV core OLP were different in both CD4 and CD8, with patients able to mount a stronger immune response than other, whose responses were weaker [***Figure 13 – A, CD4 left panel and CD8 left panel***]. This degree of heterogenicity between patients may indicate a variability in the exhaustion levels among different patients as previously reported (Chua et al., 2020, Wang et al., 2020, Aliabadi et al., 2022). Furthermore, all the patients exhibited a stronger IFN-γ production after stimulation, thus a stronger HBV-specific T cell activation compared to the unstimulated control [***Figure 13 – B, stimulated in red, unstimulated in yellow***]. Despite this, no significant differences were found in the production of IFN-γ by both CD4 and CD8 T cells, between unstimulated and stimulated sample in cohort number 1 [***Figure 13 – C, yellow and red columns respectively***].

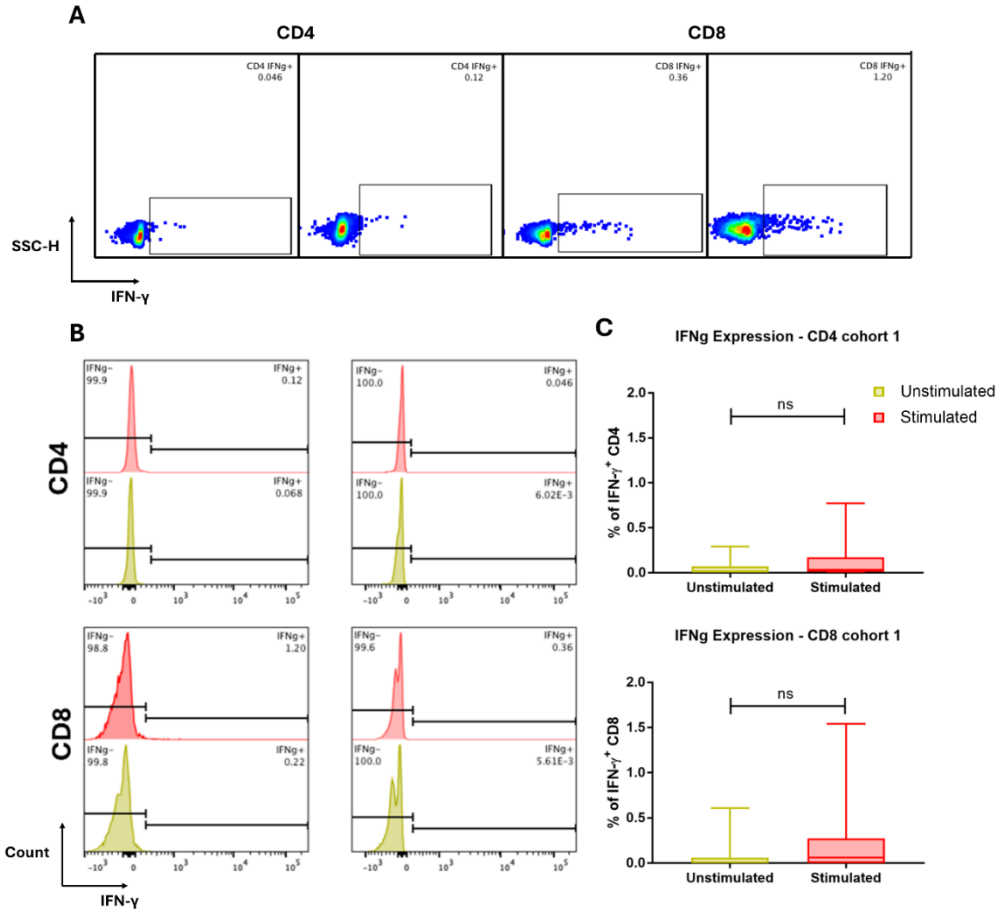

Figure 13. *HBV-specific T cells response upon stimulation*. (A) Example of IFN-γ gate from both CD4 (left) and CD8 (right) after stimulation of bulk PBMCs from CHB patients. (B) Comparison (peak) between HBV-stimulated samples (red peak) and unstimulated sample (yellow peak). (C) Comparison between the production of IFN-γ from unstimulated sample (yellow) and HBV-stimulated (red) in both CD4 T cells (upper graph) and CD8 T cells (lower graph). Graph shows the median and the 5-95th percentile.

6.4 NK cells depletion does not alter HBV-specific T cells activation in CHB patients

NK cells were removed from PBMCs as reported in [**5.2**]. The reduction rate of NK cells was reported in [***Figure 12 – C***]. NK depleted PBMCs were stimulated for 8 days with core OLP in AB media. On day 8 PBMCs were re-stimulated with 1.25 mM peptide pool, in presence of BFA for 6 hours, followed by flow cytometry staining [***Figure 14 – A***]. Despite some slight differences in the level of IFN-γ production in both CD4 and CD8 T cells, NK depletion did not improve the effector function of both HBV-specific CD4 and CD8 T cells [***Figure 14 – B, C***].

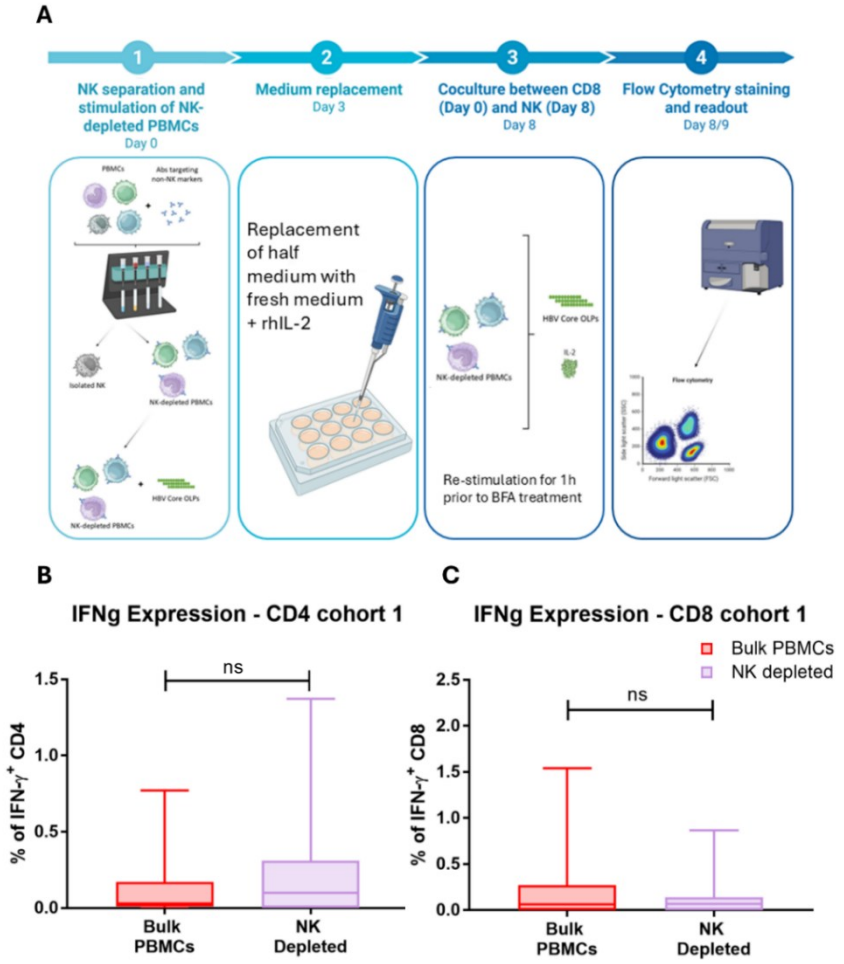

Figure 14. *NK depletion does not promote HBV-specific T expansion in patients with CHB.* (A) Schematic representation of stimulation of NK-depleted PBMCs. Briefly NK cells were removed from PBMCs by using MACS

and stimulated with core OLP for 8 days. At day 8, cells were re-stimulated in presence of BFA and stained with both surface and intracellular mAbs as reported in [**5.5**]. (B, C) Graphics of CD4 (B) and CD8 (C) T cells activation upon NK depletion.

6.5 Activated NK cells mediate the activation of HBV-specific T cells independently from PD-L1 blockade.

To further evaluate the impact of NK on mounting the expansion of HBV-specific T cells, PBMCs were stimulated in absence of NK cells for 8 days. On day 8 physiological ratios of cytokine-activated NK cells were co-cultured with peptide-expanded NK-depleted PBMCs and re-stimulated in presence of BFA for 6 hours as reported in **Figure 7**. Mann-Whitney test was performed to detect statistically significant differences. After cytokine treatment, both NK Dim and NK Bright display an increase of IFN-γ as can be observed by the gates and the histogram In **Figure 15 – *A, B*** respectively. Generally, all the NK Dim and Bright from CHB patients respond to cytokine activation by increasing the production of IFN-γ as can be observed in ***Figure 15 – C Blue and green column respectively*** and reported in (Diniz et al., 2022, Marotel et al., 2021). As indicated in ***Figure 15***, the addition of activated NK significantly boost HBV-specific T cells response in both CD4 (p-value = 0.0130) [***Figure 15 – D***] and CD8 (p-value = 0.0044) [***Figure 15 – E***] T cell by increasing their production of IFN-γ compared to PBMCs stimulated in absence of NK cells***, Figure 15 – D,E green vs purple column respectively***].

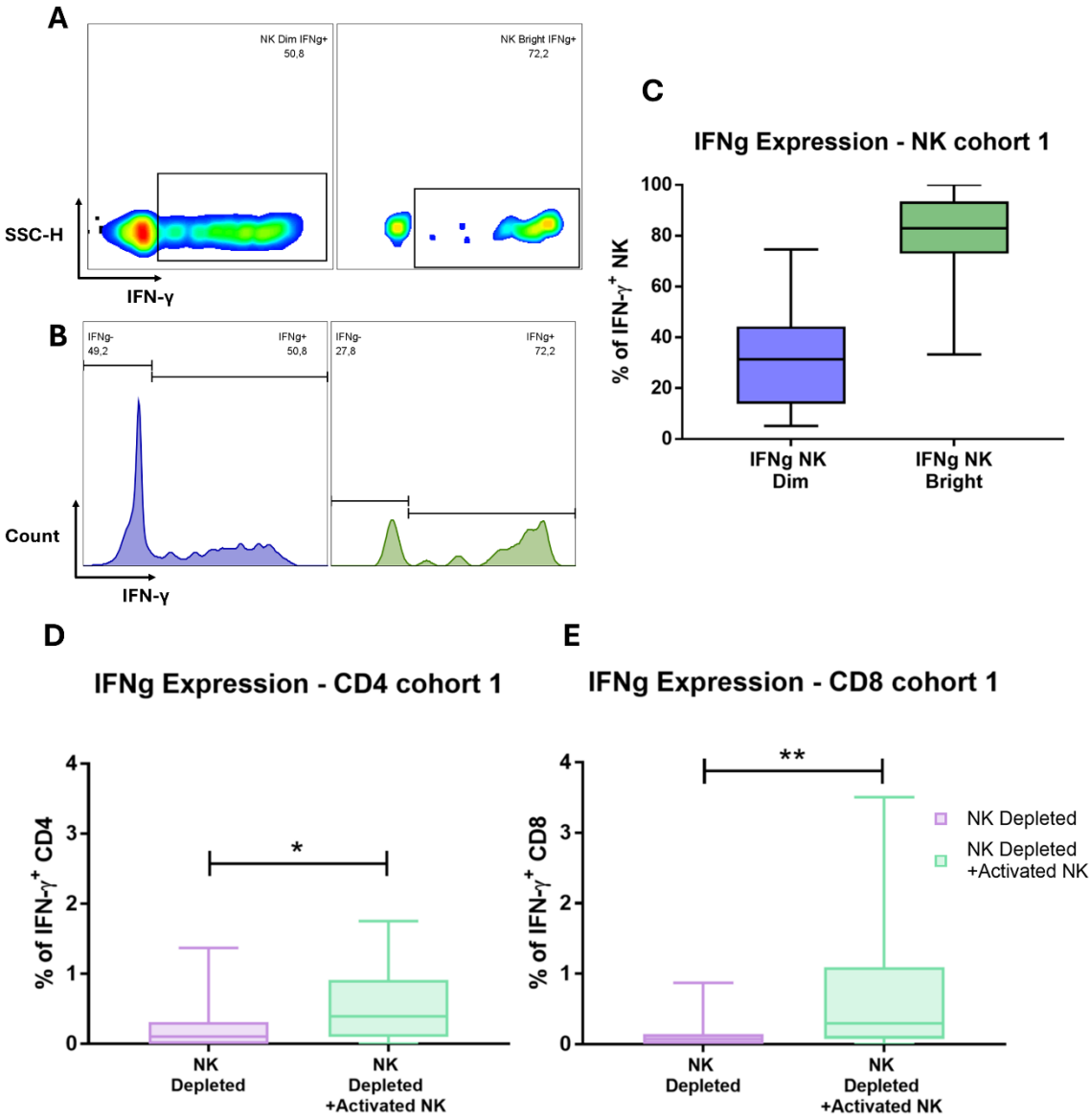

Figure 15. *Activated NK cells boost HBV-specific-T cell response.* (A – B) Both NK Dim (left) and Bright (right) increase their production of IFN-γ upon cytokine activation. (C) Level of IFN-γ expression on NK Dim (blue) and NK Bright (green) after NK activation (Graph shows the median and the 5-95th percentile). (D – E) Activated NK cells boost both CD4 (D) and CD8 (E) HBV-specific T cell response by increasing the production of IFN-γ when NK-Depleted PBMCs were re-stimulated after the coculture with autologous activated NK cells (Graph shows the median and the 5-95th percentile).

As previously reported, activated NK cells from healthy donor, mildly increase of PD-L1 expression on cellular surface (Bellucci et al., 2015). Despite this, activated NK cells from patients with CHB display a phenotype shifted towards a more exhaustion pattern, with increase expression of inhibitory checkpoint molecule such as PD-L1 compared to healthy control (p-value = 0.0339) (Diniz et al., 2022, Marotel et al., 2021) [**Figure 16 – A, B**]. Nevertheless, PD-L1 blockade on NK cells does not boost the production of IFN-γ on both CD4 and CD8 T cells [**Figure 16 – C, E respectively**], although some patients exhibit a response to the treatment [**Figure 16 – D, F for CD4 and CD8 respectively**]. As PBMCs are a highly heterogeneous population, not only NK cells express PD- L1. Thus, the effect of PD-L1 blockade on activated NK cells may be masked by T cells being inhibited by other cells such as APCs (Ye et al., 2015, Hoogeveen et al., 2020).

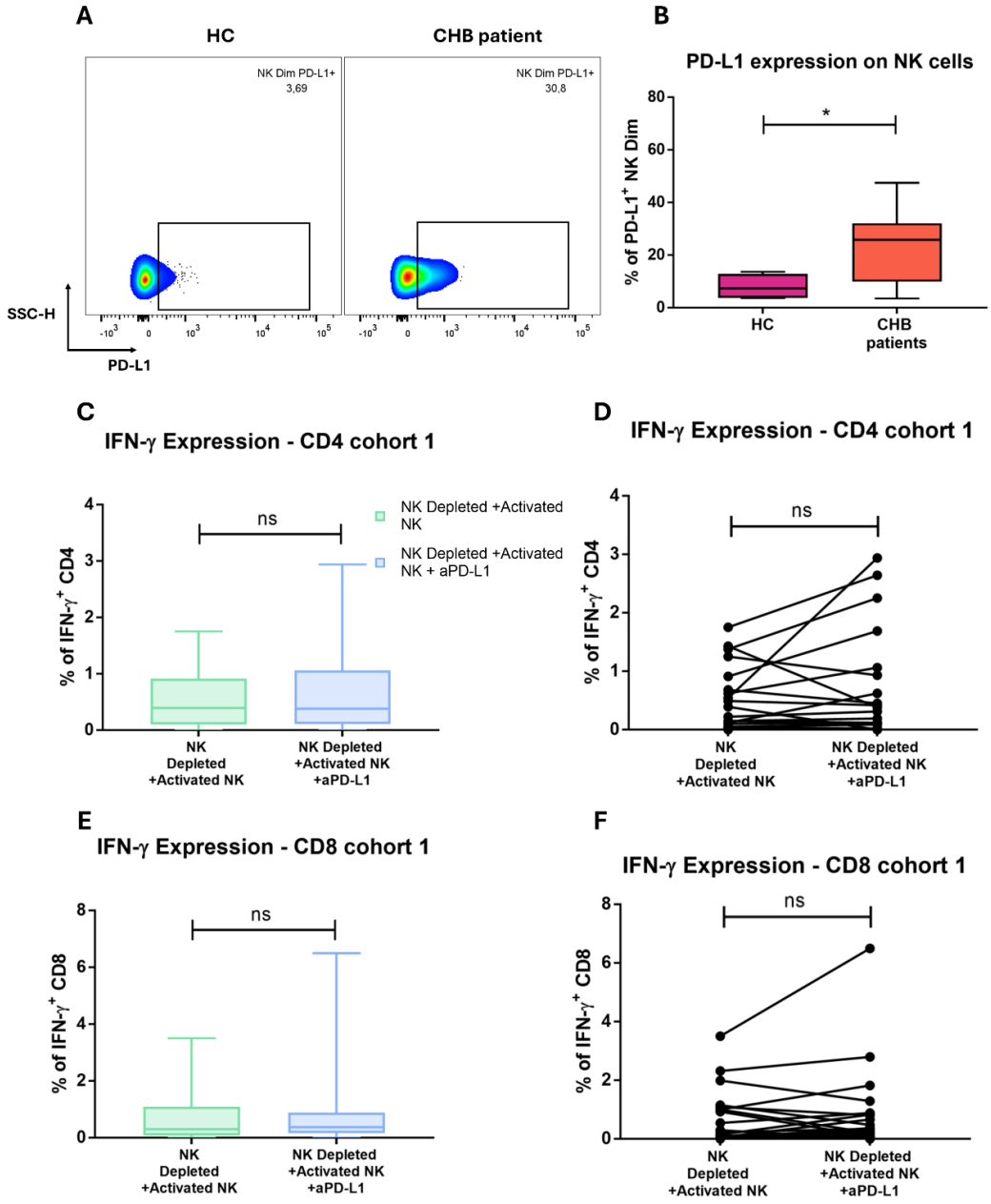

Figure 16. *PD-L1 blockade on activated NK cells has a marginal impact on HBV-specific T cell expansion.* (A – B) comparison in gates (A) and graph (B) between PD-L1 expression levels on activated NK cells between health control (HC) and CHB patients. (C – D – E – F) Comparison between the level of IFN-γ production from both CD4 (C – D) and CD8 (E – F) T cells in presence of activated NK cells without (green) and with (blue) PD-L1 blockade. Graph shows the median and the 5-95th percentile.

6.6 Activated NK cells boost HBV-specific T cell response.

To evaluate the global impact of NK cell on HBV-specific CD4 and CD8 T cell responses, data from all the four experimental conditions were plotted and analysed. Kruskal-Wallis test with Dunn's multiple comparison was performed to highlight statistical differences [***Figure 17***]. Overall, the IFN-γ production of both CD4 [***Figure 17 – A***] and CD8 [***Figure 17 – B***] T cells resulted statistically significant along the four groups. Kruskal-Wallis test indicates significant differences in the experimental groups (p-value = 0.0015, p-value = 0.0004 for CD4 and CD8 respectively). Pairwise comparisons using Dunn's post-hoc test were performed to determine the intra-groups differences.

As reported by ***Figure 17 – A***, activated NK cells boost the level of production of HBV-specific CD4 T cells [***Figure 17 – A, green column***] compared to bulk-stimulated PBMCs [***Figure 17 – A, red column***] (p-value = 0.0371). The same effect is visible comparing IFN-γ production from bulk-stimulated PBMCs and activated NK, treated with anti-PD-L1 [***Figure 17 – A, blue column***] (p-value = 0.0317). The same effect was observed for CD8 T cells where activated NK cells, and activated NK, treated with anti-PD-L1 boost the expansion of HBV-specific CD8 T cells (p-value = 0.0371 and p-value = 0.0088 respectively) [***Figure 17 – B, red column vs green and blue column respectively***]. Furthermore, activated NK cells, without or with treated with anti-PD-L1, boost HBV-specific CD8 T cell response compared to PBMCs stimulated in absence of NK cells (p-value = 0.0317 and p-value = 0.0047 respectively) [***Figure 17 – B, column green and blue vs purple column***].

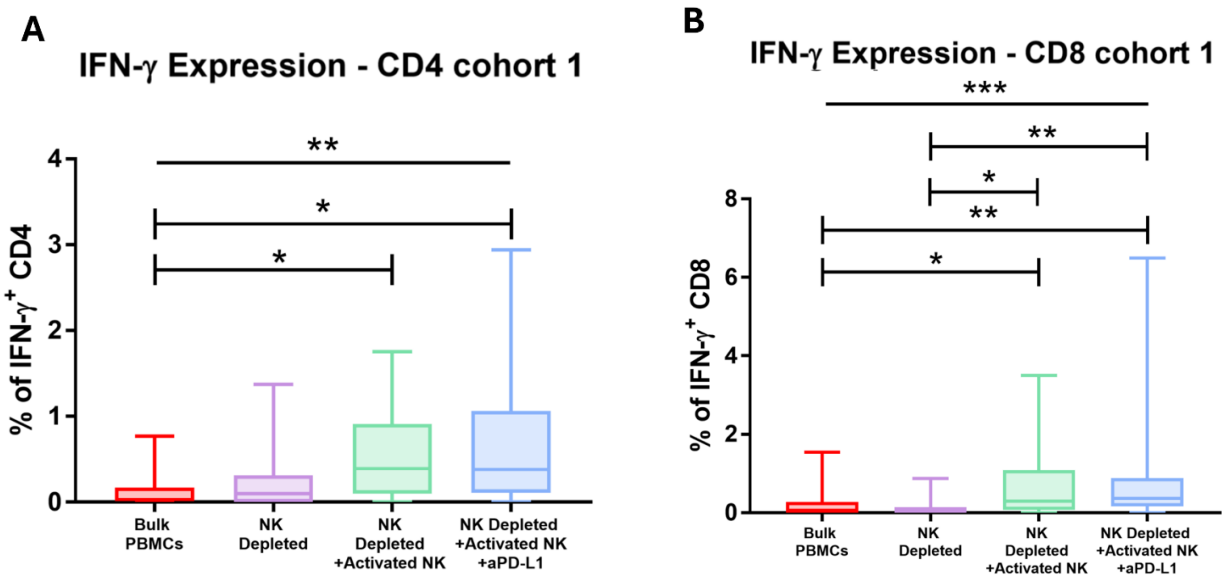

Figure 17. *Activated NK cells and PD-L1 blockade boost HBV-specific T cells response.* IFN-γ production from CD4 (A) and CD8 (B) T cells upon stimulation with HBV OLPs with, without NK and after addition of activated NK cells treated or not with anti PD-L1. Graph shows the median and the 5-95th percentile.

Objective 3: *Assessment of virological and immunological biomarkers and determination of NK cells regulation of HBV-specific T cells. Phenotypic and functional characterization of isolated NK cells following stimulation and/or checkpoint blockade.*

6.7 Identification of second cohort for further analysis of NK regulation of HBV-specific T cells

Based on the results we showed previously, we decided to further evaluate the impact of NK cells on the activation of HBV-specific T cells [***Figure 18 – A, B***]. To achieve this aim, we selected patients, previously characterize in Hannover and described in [**5.1**]. Thus, patients of the second cohort were selected based on the immune response observed during previous experiments. Particularly, in cohort number 2 were included patients with a heterogenic immune response, defined as high responders (patients with CD8 T cell IFN-γ response >1%) and low responders (patients with CD8 T cell IFN-γ response <1%)(Boni et al., 2007).

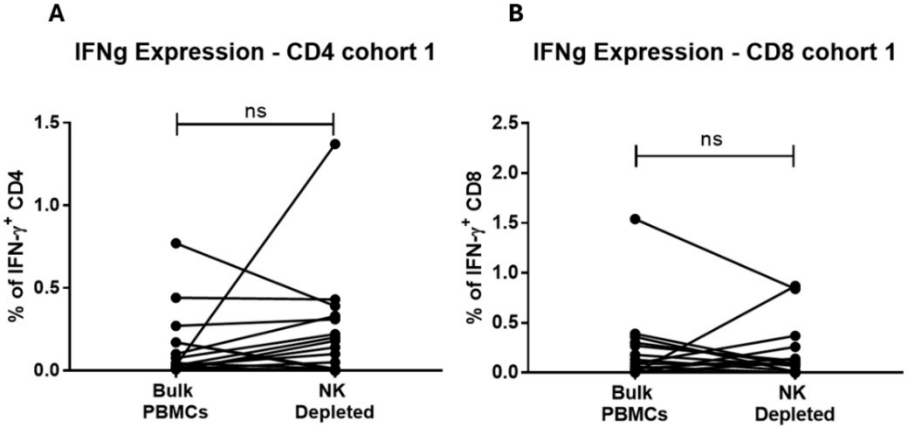

Figure 18. *Immune response after HBV stimulation with/without NK cells on cohort 1.* (A) CD4 T cells response after stimulation with/without NK cells. (B) CD4 T cells response after stimulation with/without NK cells. Each line represents the immune response of a single patient.

6.8 HBV stimulation enhances IFN-γ response in cohort 2

PBMCs were thawed and stimulated with OLPs for 8 days as reported in [**5.3**]. 6 hours prior to flow cytometry staining, cells were restimulated in presence of Brefeldin A, followed by flow cytometry readout. Mann-Whitney test was performed to detect statistically significant differences. As reported in ***Figure 19 – A***, both CD4 [***Figure 19 – A, top***] and CD8 [***Figure 19 – A, bottom***] display a high heterogenicity of production of IFN-γ upon stimulation. Moreover, both CD4 T cells and CD8 T cells significantly increase the production of IFN-γ after stimulation (p-value = 0.0441 for CD4 T cells and p-value = 0.0019 for CD8 T cells) compared to the unstimulated control [***Figure 19 – B, C respectively***].

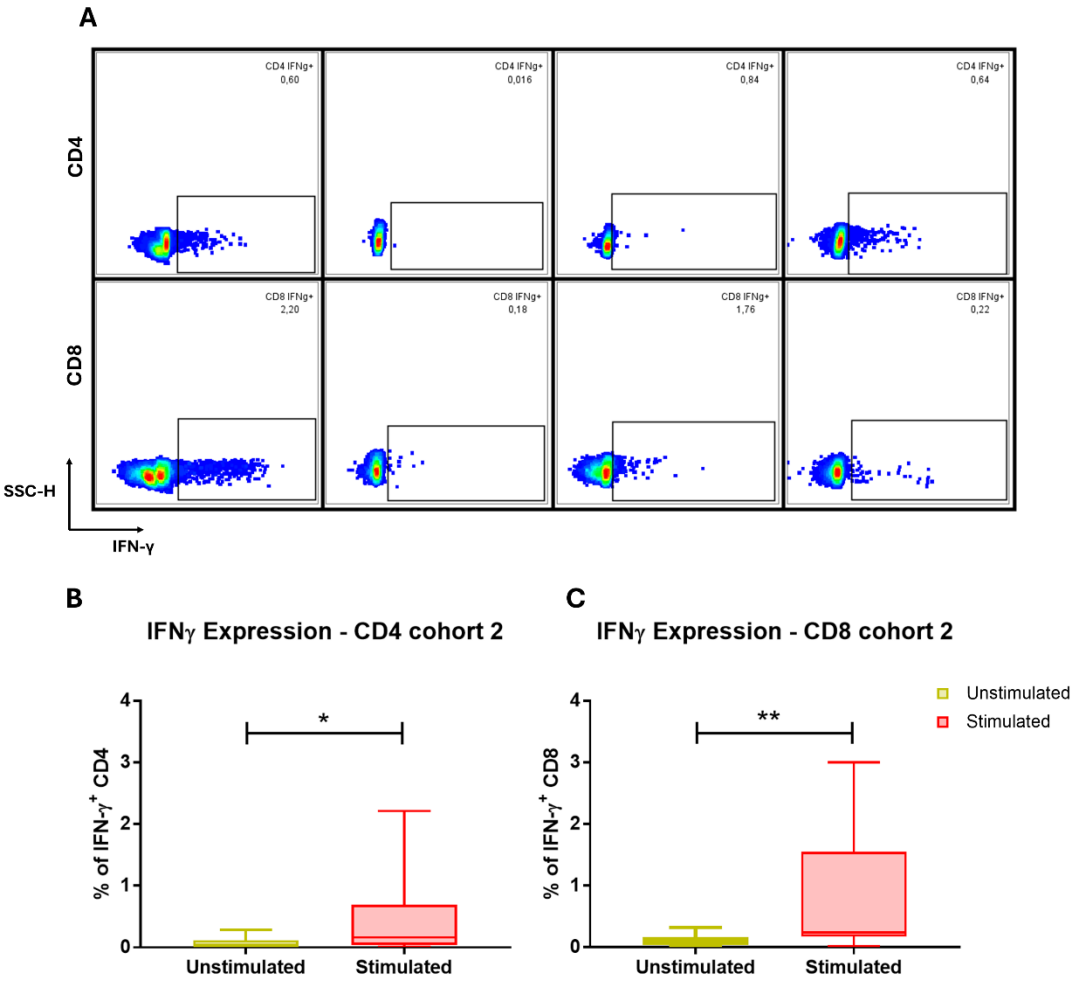

Figure 19. *HBV stimulation boost IFN-γ production in a heterogeneous way both CD4 and CD8 T cells*. (A) Gates representing IFN-γ production from both CD4 (top) and CD8 (bottom) T cells upon stimulation with HBV OLPs. (B, C) Graph showing the median and the 5-95th percentile of IFN-γ response in unstimulated and stimulated sample.

6.9 NK cells regulation of HBV-specific T cells depends on patients' responsiveness to HBV stimulation

To evaluate the global impact of NK cell on HBV-specific CD4 and CD8 T cell responses, Kruskal-Wallis test with Dunn's multiple comparison was performed to highlight statistical differences [***Figure 20***]. Overall, the IFN-γ production of both CD4 [***Figure 20 – A***] and CD8 [***Figure 20 – B***] T cells resulted statistically significant along the four groups. Kruskal-Wallis test highlight significant differences in the experimental groups (p-value = 0.0010, p-value = 0.0049 for CD4 and CD8 respectively). Pairwise comparisons using Dunn's post-hoc test were performed to determine the intra-groups differences.

In detail, by stimulating PBMCs in absence of NK cells, a significant decrease of HBV-specific T cells can be observed (p-value = 0.0204 for CD4 and p-value = 0.0242 for CD8). This phenotype is then recovered if activated NK were added prior to the restimulation (p-value = 0.0016 for CD4 and p-value = 0.0271 for CD8), independently from PD-L1 blockade or not as in cohort number 1 [***Figure 17***] . Moreover, some patients show a more pronounced IFN-γ response compared to its corresponding stimulation of bulk PBMCs, but these differences do not result statistically significant.

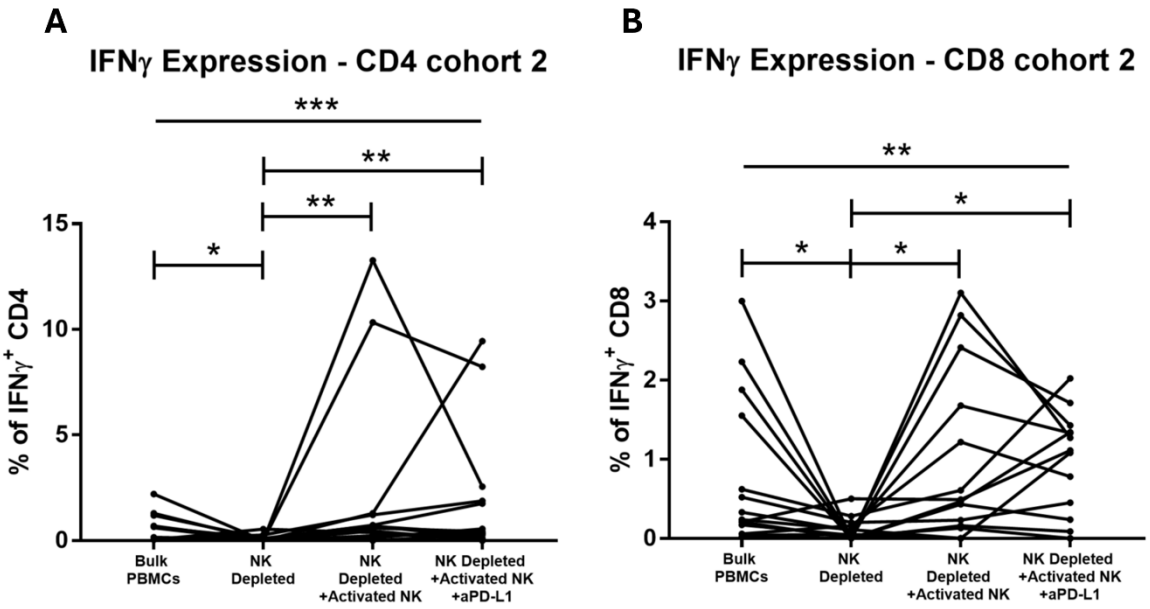

Figure 20. *impact of HBV stimulation on PBMCs cultured in presence of NK, in their absence and with cytokine activated NK cells without and with PD-L1 blockade*. (A) CD4 T cell IFN-γ production after stimulation with different NK cell conditions. (B) CD8 T cell IFN-γ production after stimulation with different NK cell conditions. IFN-γ were detected by intra-cytokine staining.

Analysis of the data from cohort number two revealed the presence of two distinct populations among the considered cohort. Patients exhibiting a higher CD8 immune response lost their immune activity following NK cell depletion. By contrast, patients with a cellular response lower than 1% either benefited from NK cell depletion or showed no detectable chang in their immune response. Thus 2 different groups were obtained, those with an INF-γ production above 1% were classified as top responders [***Figure 21 – A for CD4 and C for CD8***], whereas those with an IFN-γ production below 1% were classified as low responders [***Figure 21 – B for CD4 and D for CD8***].

Kruskal-Wallis test was performed to detect statistically significant differences among the four groups. As reported in ***Figure 21***, both top responders and low responders display significant differences among the for groups for either CD4 top responders (p-value = 0.0174) [***Figure 21 – A***], CD4 low responders (p -value = 0.0341) [***Figure 21 – B***], CD8 top responders (p-value = 0.0096) [***Figure 21 – C***] and CD8 low responders (p-value = 0.0485) [***Figure 21 – D***].

Furthermore, the results from top responders display an abrogation of immune activation of HBV-specific T cells upon depletion of NK cells in both CD4 (p-value = 0.0206) [***Figure 21 – A***] and CD8 (p-value = 0.0137) [***Figure 21 – C***]. This effect was not observed in the cohort of low responders, indicating that the better immune response to stimulation may be the result of a synergic crosstalk between HBV-specific T cells and NK cells.

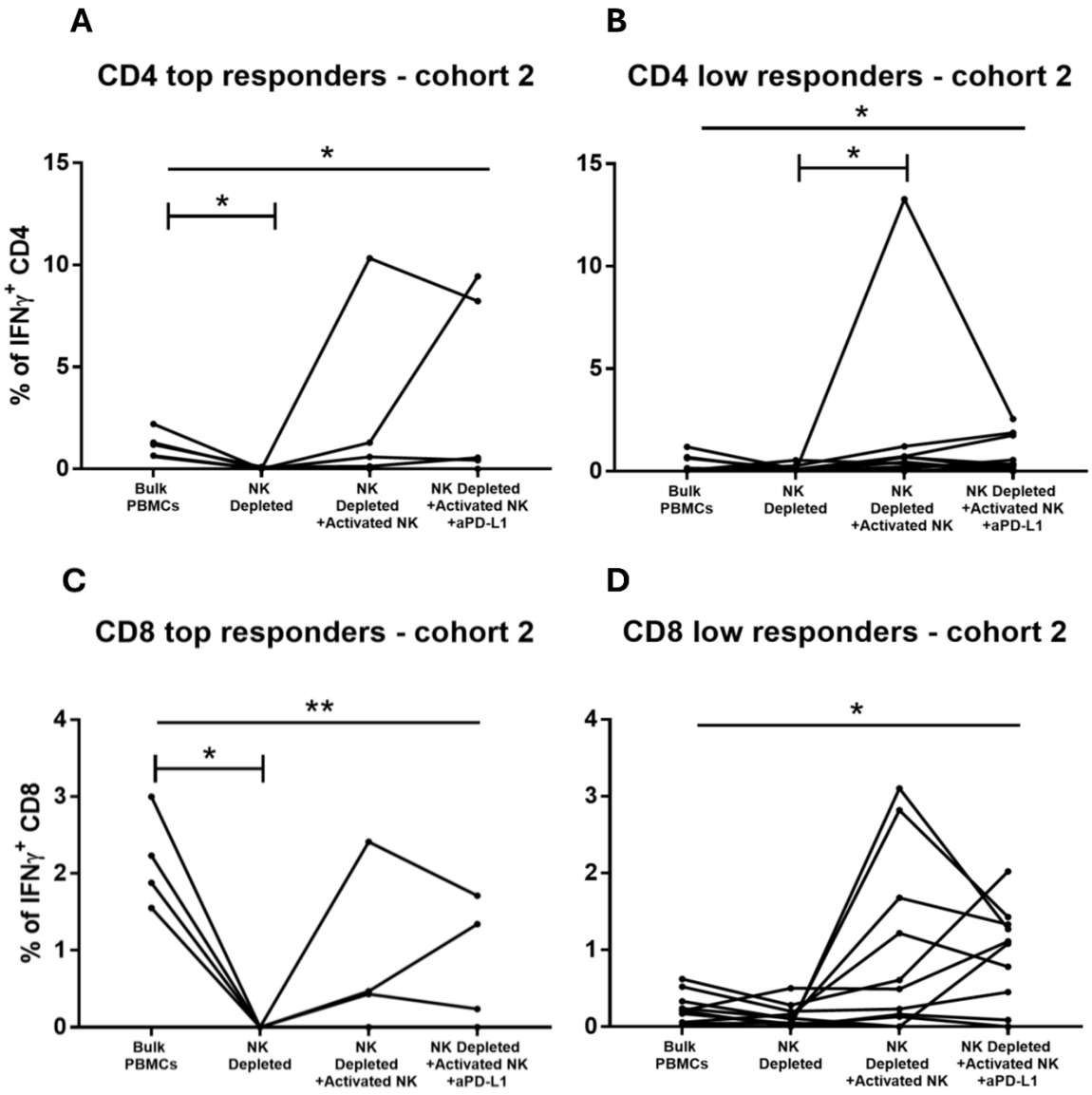

Figure 21. *HBV-specific immune response among top responders and low responders during CHB.* (A, B) CD4 T cell response to stimulation with different NK conditions from top responders (A) and low responders (B). (C, D), CD8 T cell response to stimulation with different NK conditions from top responders (C) and low responders (D)

6.10 CD8 T cell exhaustion levels associates with enhanced NK cell activation

To further evaluate NK regulation of HBV-specific T cells, a correlation analysis between CD8 T cell and NK Dim was performed by using Spearman correlation analysis test. ***Figure 22*** shows the correlation heatmap between CD8 T cells and NK dim. As reported, the level of IFN-γ production of NK cell upon cytokine stimulation negatively correlates with the level of HBV-specific CD8 T cells (p-value = 0.03337, ρ = -0.5559) [***Figure 22 and Figure 23 – A***]. This may indicate a compensatory mechanism in which the presence of highly exhausted T cells hijacks the immune response towards a more innate response, explaining the increased IFN-γ production observed in NK cells (Cornberg et al., 2013, Stegmann et al., 2010). These findings were further supported by the positive correlation between the expression of PD-L1 on NK dim and the level of NK activation (p-value = 0.0322, ρ = 0.5607) [***Figure 22 and Figure 23 – B***].

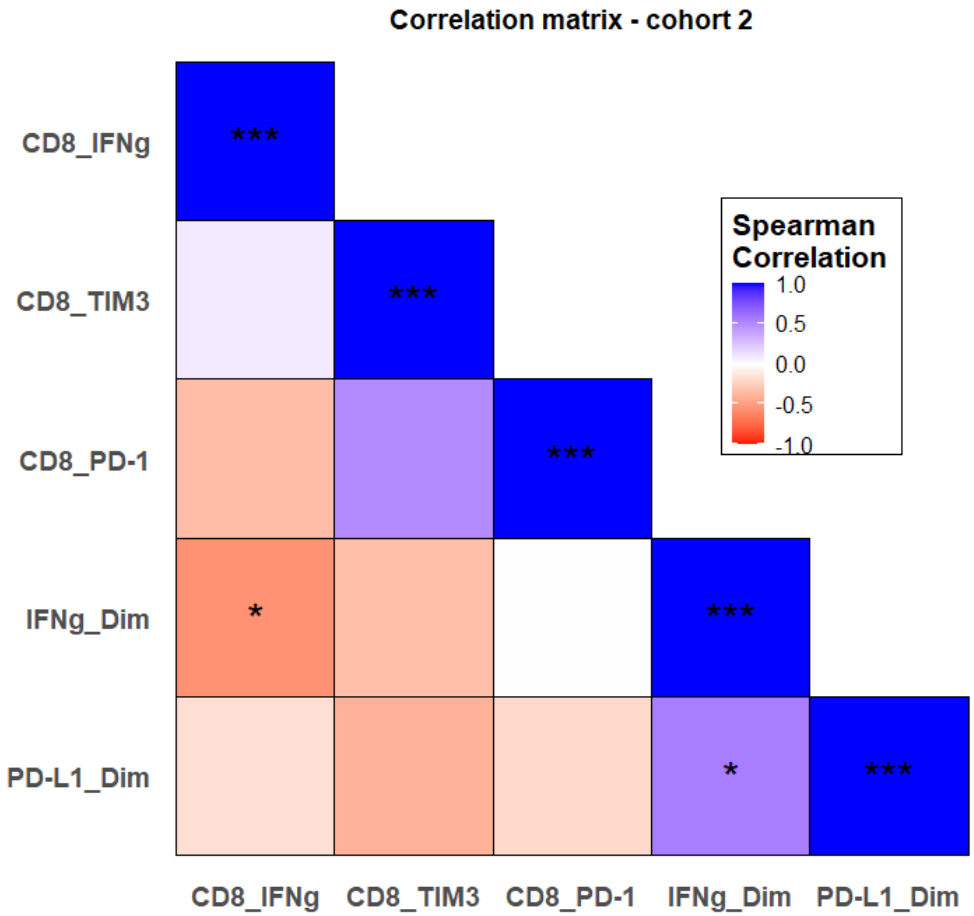

Figure 22. *Correlation matrix between NK dim and CD8 T cell.* NK activation level negatively correlates with the level of IFN-γ expression from HBV-specific CD8 T cells.

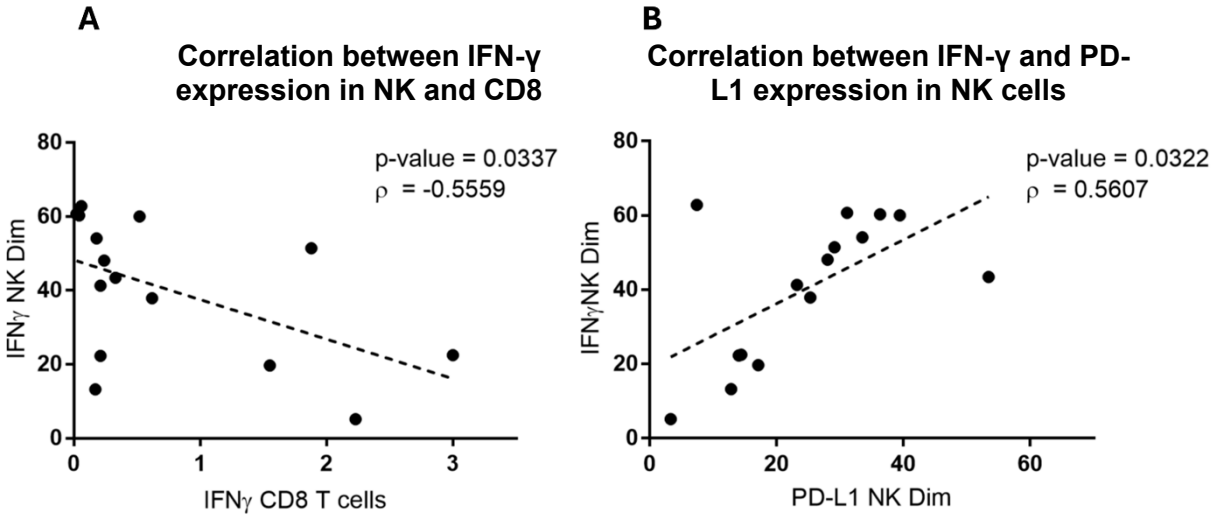

Figure 23. *Spearman correlation between the expression of IFN-γ and PD-L1 on NK cells and CD8 T cells.* (A) correlation between the expression of IFN-γ on NK Dim and CD8 T cells. (B) correlation between the expression of IFN-γ and PD-L1 on NK Dim.

6.11 Highly activated NK cells display an inhibitory phenotype

Dimensionality reduction has been performed on NK cells as reported in [**5.6**]. Briefly NK cells were gated based on the expression of CD56, after doublets and dead cells exclusion. Gated NK cells were concatenated and tSNE plot were performed with a perplexity value of 30. Clustering was performed by FlowSOM algorithm. Dimensionality reduction identifies a cluster of NK Bright cells [***Figure 24 – Pink***] strongly positive for IFN-γ. Simultaneously, this cluster of cells resulted to be positive for PD-L1, CD57 and NKG2A, indicating an inhibitory phenotype, capable of suppressing the response of other immune cell subsets (PD-L1, NKG2A, TRAIL), while exhibiting an highly activated state (IFN-γ, NKG2D, CD57) (Kaulfuss et al., 2023, Marotel et al., 2021, Nielsenet al., 2013, Diniz et al., 2022). This phenotype has been also partially identified in a proportion of NK Dim [***Figure 24 – Black***]. This phenomenon further supports the mutual compensatory mechanism between CD8 T cells and NK cells, described above in the second cohort [**5.1**].

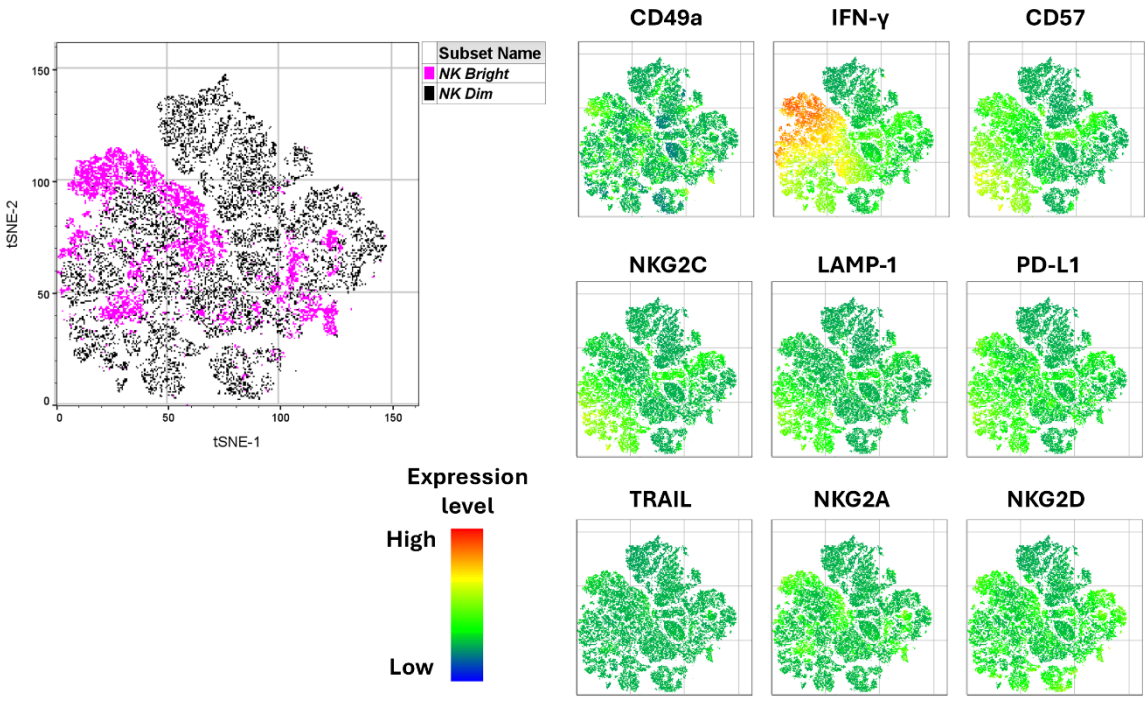

Figure 24. *Dimensionality reduction on NK cells.* NK cells were clustered as NK Dim (Black) and NK Bright (Pink) based on the expression level of CD56 and CD16. Both NK Dim and Bright display an activated phenotype, with high expression of IFN-γ and NKG2D. At the same time, activated NK cells boost their expression of inhibitory ligand such as NKG2A and PD-L1.

7. Discussion and further studies

This work aimed to better understand how NK cells regulate the HBV-specific T cell response in CHB patients, in order to provide immunological insight that can guide future intervention strategies. The main take-home message is that NK depletion impairs HBV-specific CD4 and CD8 T cell response, suggesting that immunomodulation of the NK compartments could potentially be considered to design future therapies.

HBV is a non-cytopathic virus and generally leads to a self-resolving acute infection (Glebe et al., 2013; Yuen et al., 2018). However, in unvaccinated children and immune-deficient patients, this infection can progress towards a chronic state due to uncontrolled viral antigen production and presentation to immune system cells. Conventional NK cells have been shown to play a crucial role in this process, upregulating inhibitory ligands on their surface to inactivate T cells response (Yuen et al., 2018; Khanam et al., 2021; Yang et al., 2019). Among these, PD-L1 is one of the most well characterised: through the interaction with PD-1, expressed on lymphocytes surface, it represses any antigen-specific CD4 and CD8 T cells response (Diniz et al., 2022). Indeed, when the PD-L1 cascade is suppressed, there is a rescue the proliferation of HBV-specific T cells observed with an increase of IFN-γ production, in murine models (Diniz et al., 2022). This work aimed to better understand how NK cells regulate the HBV-specific T cell response. To achieve this objective, two different cohort of patients were characterized in the study. PBMCs from CHB patients were stimulated with HBV core OLPs in presence or not of NK cells, or in presence of activated NK cells without/ with PD-L1 inhibitors. We selected specifically core OLPs due to their higher immunogenicity compared to other HBV proteins (e.g. HBsAg, HBeAg), as previously described (Aliabadi et al., 2022; Diniz et al., 2022).

Despite being more immunogenic, HBc stimulation slightly increase the activation level of both CD4 and CD8 T cells in cohort 1. This phenomenon

further highlights the exhaustion level of CHB patients, with CD4 and CD8 T cells barely reacting to the stimulation (Boni et al., 2007, Aliabadi et al., 2022). The levels of activation of HBV-specific T cells remain unchanged after NK depletion prior stimulation and restimulation after 7 days of culture. Despite this, addition of cytokine activated NK cells improved HBV-specific T cells response in both CD4 and CD8 T cells, indicating that activated NK cells may be beneficial for immune response recovery. Nevertheless, activated NK cells from CHB patients shows an increase production of PD-L1 in both NK Bright and Dim compared to activated NK cells from healthy control. Yet, PD-L1 blockade on activated NK cells does not improve the production of IFN-γ from HBV-specific T cells. Since PBMCs are a highly heterogeneous population, PD-L1 is not only confined to NK cells. Thus, the effect of PD-L1 blockade on activated NK cells may be masked by T cells being inhibited by other cells such as APCs (Ye et al., 2015, Hoogeveen et al., 2020).

The outcome of cohort number 1 demonstrates that patients exhibiting a robust immune response following stimulation either fully or partially lose their capacity to mount an effective immune response subsequent to NK depletion on both CD4 and CD8 T cells. We decided to explore and potentially validate these preliminary results in another patient's cohort. Patients within the second cohort were selected based on the immune response after HBV stimulation observed during prior experiments. This response was defined as either high (patients with a CD8 T cell IFN-γ response >1%) or low (patients with a CD8 T cell IFN-γ response <1%), as previously described (Boni et al., 2007). Overall, following the stimulation protocol, we observed that PBMCs from cohort 2 were characterized by an increased responsiveness to antigenic stimulation, with a level of IFN-γ production upon stimulation significantly higher compared to the unstimulated control in both CD4 and CD8.

When comparing the two groups of patients (high vs low HBV-specific T response), IFN-γ production on T cells significantly decrease in the high responder group after NK depletion. Moreover, IFN-γ response were partially

recovered after addition of activated NK cells. This phenomenon was not observed in the low responder group, indicating that NK cells may be fundamental to mount a proper immune response after antigenic stimulation. Moreover, NK activation level negatively correlates with the level of IFN-γ production on HBV-specific T cells and positively correlates with the level of PD-L1 expressed on NK cells. These findings, suggest a compensatory mechanism between NK cells and HBV-specific T cells. Highly exhausted T cells may hijack the immune response towards a more innate response, explaining the increased IFN-γ production observed in NK cells (Cornberg et al., 2013, Stegmann et al., 2010). Dimensionality reduction analysis, further characterize the phenotype of activated NK cells revealing a two population of NK cells (one of NK Bright and one of NK Dim) strongly positive for PD-L1, CD57 and NKG2A, indicating an inhibitory phenotype, capable of suppressing the response of other immune cell subsets (PD-L1, NKG2A, TRAIL), while exhibiting an highly activated state (IFN-γ, NKG2D, CD57).

These findings paved the way for future research to more comprehensively elucidate the molecular mechanisms underlying NK cell-mediated regulation of HBV-specific T cells, in order to design future possible therapies. Future prospectives for the presented study include transcriptomic profile on activated NK cells to better clarify how NK cells modulate the functionality of HBV-specific T cells during CHB.

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