UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE BIOLOGIA ANIMAL

Immunological Characterization of Mucosal-Associated Invariant T (MAIT) Cells in chronically Hepatitis B infected patients

Tiago Nuno Ribeiro Alves

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Dissertação orientada por:
Antony Chen
Maria Gabriela Rodrigues

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Resumo

A Organização Mundial de Saúde considera a doença da Hepatite B como a oitava causa de mortalidade a nível mundial e estima que aproximadamente 400 milhões de pessoas estarão infetadas nos dias de hoje. O vírus da hepatite B é caracterizado por ter um tamanho reduzido, possuir uma conformação genómica baseada em 4 "*open reading frames*" (ORFs), ser transmitido de forma singular e com um modo ação muito efetivo. Pacientes infetados com o vírus da hepatite B, dependendo da idade em que foram infetados, podem ser diferenciados em pacientes agudos ou devido ao historial de progressão da doença ser considerados pacientes crónicas, tendo um risco elevado de virem a contrair uma de duas patologias no futuro – cirrose hepática e cancro do fígado. Actualmente existem dois regimes terapêuticos utilizados contra a hepatite B crónica, sendo estes "*Nucleos(t)ide analogues"* (NUCs) e tratamentos a base de interferões. Contudo, apesar de promoverem uma cura funcional, estes tratamentos são considerados ineficazes a longo prazo contra a infeção, uma vez que não conseguem erradicar o ADN viral (cccADN) dos hepatócitos infetados e o seu consumo é imprescindível a partir do momento do seu diagnóstico.

À semelhança de outras doenças virais crónicas, como HIV e Hepatite C também pacientes infectados com o virus da Hepatite B devido á exposição do mesmo por tempo indeterminado até ao final da vida, ficam sujeitos a uma continua disfunção a nivel celular. As células T, membros activos do nosso sistema imunitário, após combate contínuo perante a infeção, acabam por perder gradualmente a sua função contra o virus e acabam por entrar num estado de exaustão. Este processo está documentado em vários estudos científicos e sugere que não é necessariamente o grau e intensidade da infeção viral que aumenta, mas sim o próprio sistema imunitário que, por estar em constante esforço, acaba por ser ineficaz em erradicar o virus do corpo humano. No entanto, a comunidade científica está a direcionar esforços para a criação de novas medidas terapêuticas, que passam por fazer um targeting viral e utilizar mecanismos de imunoterapia que promovam a erradicação do vírus via a eliminação do ADN viral das células infetadas.

Como exemplo, as células *"mucosal-associated invariant T"* (MAIT) são vistas como uma possível nova abordagem imunoterapêutica contra o vírus da hepatite B. As células MAIT constituem um subsistema das células T, sendo caracterizadas por serem inatas, não convencionais e reconhecerem metabolitos de riboflavina microbiana (Vitamina B2). Este reconhecimento acontece no seguimento da secreção de riboflavina microbiana por parte de diversas espécies bacterianas e fungos e são apresentadas as células MAIT por intermédio da molécula não-polimórfica do complexo de histocompatibilidade principal ("*major histocompatibility complex*", MHC) classe I (MR1). Estas células estão ainda evolutivamente conservadas entre várias espécies e podem ser encontradas em larga escala nos tecidos da mucosa humana, sangue periférico e fígado, existindo uma correlação direta com a acumulação por parte destas células em locais de infeção e fornecimento de proteção contra infeções. Tendo por base a sua localização celular e a sua frequência no fígado, é sugerido que em ambientes de exaustão imunitária como é o caso da hepatite B, as células MAIT podem subsistir e estar funcionalmente ativas, imitando o papel imunitário das células T contra a infeção.

Adicionalmente, as células MAIT dispõem de diversas funções effectoras quando estimuladas e, consequentemente, activas. A produção de citoquinas pro-inflamatórias, tais como IFN-γ e TNF-α, assim como degranulação, citotoxicidade, proliferação celular (marcador Ki-67) e respostas dependentes/independentes da presença do MR1, previamente referido, são algumas das funções effectoras relevantes levadas a cabo por estas células e que influenciam o seu desempenho junto do corpo humano. É importante mencionar que as células MAIT só recentemente comecaram a ser estudadas de uma forma mais abrangente estando associadas a diferentes patologias, como é o caso deste projecto, no qual nos focamos no papel destas células no contexto do virus da hepatite B. Contudo, na sua grande maioria, os marcos existentes referentes á função celular destas células

remetem para infeções bacterianas, uma vez que estas células activamente reconhecem metabolitos produzidos por espécies bacterianas, nomeadamente no caso da *Eschericia coli*.

O foco do nosso projecto passou pela caracterização de células MAIT, previamente isoladas de amostras de PBMCs de dadores saudáveis e doentes crónicos infetados com hepatite B, com o intuito de comparar o seu fenótipo, assim como a sua resposta funcional aquando estimulação com citoquinas recombinantes, neste caso as interleucinas IL-12 e IL-18. Além disso, estas células foram ainda avaliadas quanto à expressão de três painéis de anticorpos diferentes: 1) "*Checkpoint Inhibitors"* (PD-1, TIM3 e LG3), 2) "*Activation Markers"* (CD25, CD38, CD69 e HLA-DR) e 3) "*Pro-Inflammatory Cytokines*" (IFN-γ, TNF-α e IL-2), e analisadas através de citometria de fluxo. Ao mesmo tempo, o perfil expressivo de citoquinas e quimiocinas das células MAIT foi examinado na presença de uma linhagem celular humana (*B lymphoblast - C1R*) e aquando estimulação com um composto agonista do MR1 (*Diclofenac*). As células MAIT de 3 pacientes saudáveis e 3 pacientes crónicos infectados com o virus da hepatite B foram testadas. É sabido que os marcadores avaliados são expressos pelas células MAIT em pacientes saudáveis. Contudo, o intuito de avaliarmos os mesmos em pacientes infectados passou por perceber se, na presença de uma infeção crónica, estas células conseguiriam permanecer funcionais. Ainda assim, foi necessário perceber se o virus ou o tempo de infeção, que pode variar de paciente para paciente, promove uma alteração na frequência das células MAIT e na produção dos diversos marcadores testados.

Embora os nossos dados preliminares sejam indicativos de que as células MAIT são ativamente funcionais em pacientes crónicos com hepatite B, comparativamente com os pacientes saudáveis testados, no futuro é necessária a recriação destas experiências num maior número amostral, uma vez que o nosso valor de *N* neste projeto é limitado. Não obstante, foi possível concluir com este projeto que células MAIT de pacientes infectado com HBV conseguem ser estimuladas e activadas pelas duas citoquinas recombinantes anteriormente referidas, IL-12 e IL-18, produzindo diversas citoquinas necessárias para que a sua actividade citotóxica seja equiparável as células MAIT de pacientes saudáveis. No entanto, foi verificado que a frequência dos marcadores de exaustão testados aparenta ser superior em pacientes crónicos. Contudo, seria importante testar células MAIT provenientes de amostras hepáticas de pacientes crónicos, com o objetivo de comparar com os resultados obtidos neste projecto, uma vez que o fígado é um órgão de alto risco em caso de infeção por hepatite B. Não descurando, os dados obtidos neste projeto mostram que as células MAIT poderão ter um papel imunoterapêutico promissor contra o vírus da hepatite B em combinação com terapias já existentes.

O estudo cientifico realizado no âmbito de tese de mestrado demonstrou que as células MAIT não seguem o caminho clássico de exaustão celular e conseguem reter parcialmente a sua funcionalidade em ambientes desfavoráveis, como é o caso do fígado e sangue de pacientes crónicos com hepatite B. Por conseguinte, isto é indicativo de que as células MAIT são um alvo promissor quanto ao seu uso no âmbito da imunoterapia em infeções virais crónicas e no tratamento tumoral onde o local activo da infeção seja um ambiente resiliente. Para concluir, torna-se imeprativo mencionar que existem diversas questões para as quais não existem ainda respostas evidentes relativamente ao papel activo das células MAIT. Contudo, sabemos que estas células representam um facção substancial de células T especifícas circulatórias e teciduais no corpo humano. Deste modo, um maior número de estudos ciêntificos são fundamentais para compreender a contribuição das células MAIT para uma resposta imunitária contra o virus do HBV e outras infeções virais.

Palavras-Chave: *Hepatite B, Células MAIT, Imunoterapia*

Abstract

Hepatitis B is considered by the World Health Organization (WHO) as the eight most frequent cause of mortality worldwide, with approximately 400 million infected people and without a definitive cure available. The main hallmarks of HBV are its small size, genomic conformation, transmission mechanism and mode of action. Chronic HBV patients present higher risk of disease progression to state of liver cirrhosis and hepatocellular carcinoma (HCC).

Currently, there are two treatment regimens for chronic HBV infection – Nucleos(t)ide analogues (NUCs) and IFN-based therapies – that when used can stop viral replication and induce the innate immune system to fight and control the infection, even though with less than 10% cure rate. However, these treatments are still ineffective in eradicating HBV infected hepatocytes, since its cccDNA genome formation is considered ultra-stable. Nonetheless, viral targeting and immunotherapeutic approaches are emerging, since a therapy that involves adaptive immunity (T cells) is required that can control the infection and promotes viral clearance from infected cells. Unfortunately, in chronically infected patients, HBV specific T cells are non-functional (exhausted), hence we looked into mucosal-associated invariant T (MAIT) cells because these cells are capable to sustain effector function despite having upregulated PD-1 exhaustion marker and are present in high frequency within the liver. With these qualities, MAIT cells are viewed as a possible new cell-based immunotherapeutic approach against HBV infection.

MAIT cells are unconventional innate-like T cells subset characterized for recognizing microbial riboflavin metabolites (Vitamin B_2) secreted by numerous bacteria and fungi species presented via the non-polymorphic major-histocompatibility complex (MHC) class-I related molecule (MR1). Besides being evolutionary conserved among species, MAIT cells also play a protective role that can be directly related to a highly abundant frequency in human mucosal tissues, peripheral blood and liver, as well as an increased tendency to accumulate at the infection sites. Based on these cells' localization and high frequency within the liver, it is suggested that in an HBV infected liver environment, MAIT cells can still be actively functional and can be developed into a therapeutic cure for chronic HBV infection.

In this project, we characterized the phenotypic signature of MAIT cells isolated from healthy and chronically infected HBV patients' PBMCs as well as their functional response upon stimulation with IL-12 and IL-18 recombinant cytokines. MAIT cells were evaluated regarding the expression of three different antibody panels – 1) checkpoint inhibitors, 2) activation markers and 3) pro-inflammatory cytokines – and analyzed using a FACS flow cytometer. Furthermore, MAIT cell cytokine and chemokine expression profile were assessed, when in contact with human B lymphoblast antigen presenting cell line and while stimulation with a MR1 agonistic compound (diclofenac).

Although our preliminary data shows that MAIT cells from chronic HBV patients are functional in *ex vivo* stimulation, it will be of interest to further test the developed assays with additional human PBMC samples, since our sample size in this project is limited. Also, it would be of high relevance to test MAIT cells retrieved from chronic HBV patients' liver samples and compare the data with our results. Nonetheless, the data obtained in this project shows that MAIT cells are functional, even though these cells do not possess a TCR, which leads to the unrecognition of recognize infected hepatocytes. Nonetheless, MAIT cells are considered as a promising cell-based therapeutic approach that could be used against HBV in combination with the already existent therapies.

Keywords: *Hepatitis B virus, MAIT cells, Immunotherapy*

Table of Contents

Acronyms

5-A-RU - 5-amino-6-D-ribitylaminouracil

5-OE-RU - 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil

5-OP-RU - 5-(2-oxopropylideneamino)-6-Dribitylamonouracil

6-FP - 6-formylpterin

Ac-6-FP – Acetyl 6-formylpterin

ALT - Alanine aminotransferase

APC – Antigen presenting cell

cccDNA - Closed circular DNA

CTLs - Cytotoxic Lymphocytes

ER – Endoplasmic reticulum

CCR - C-C chemokine receptor

CD – Cluster of differentiation

CXCR - CXC chemokine receptors

CHB – Chronic Hepatitis B

DC – Dendritic cell

DCF - Diclofenac

ER - Endoplasmic reticulum ETV - Entecavir

GM-CSF - Granulocyte-macrophage colony stimulating factor

Gnly - Granulysin

GrzA/B – Granzyme A/B

HBcAg – Core antigen

HBeAg - Hepatitis B e-antigen

HBsAg - Hepatitis B surface antigens

HBx - Hepatitis B Virus X protein

HBV – Hepatitis B virus

HCC – Hepatocellular carcinoma

HCV - Hepatitis C virus

HLA-DR - Human leukocyte antigen-antigen D related

IC - Immune complex

IFNα - Interferon α

IFNγ – Interferon γ

IL– Interleukin

IR - Inhibitor receptor

ISGs - Interferon-stimulated genes

LAG3 - Lymphocyte activation gene-3

LAM – Lamivudine LPS - Lipopolysaccharide

MAIT - Mucosal-associated invariant T

MDR1 - Multidrug resistance protein 1

MHC - Major-histocompatibility complex

MR1 – MHC-related protein-1

MAIT - Mucosal-associated invariant T

MVA - Modified vaccinia virus Ankara

NCPT - Sodium taurocholate co-transporting polypeptide

NK – Natural killers

NUCs - Nucleos(t)ide analogues

ORF – Open reading frame

PBMCs - Peripheral blood mononuclear cells

PD-1 - Programmed cell death protein 1

PEG-IFNα - Pegylated interferon-α

pgRNA – Pre-genomic viral RNA

PMA - Pphorbol myristate acetate

Pol/RT - Polymerase/reverse transcriptase

Pre-S/S, Pre-S2/M and Pre-S1/L – Small, medium and large HBV proteins

Prf - Perforin

PRR – Pattern recognition receptor

RcDNA – Relaxed-circular DNA

SVP – Sub-viral particle

TCR - T cell receptor

TDF - Tenofovir

Th - T helper

TIM3 - T-cell immunoglobulin domain and mucin domain 3

TLR - Toll-like receptor

TNFα - Tumor necrosis factor α

WHO - World Health Organization

Chapter 1 – Introduction

1.1 Molecular Virology of Hepatitis B Virus

Besides being one of the smallest virus present in nature, hepatitis B virus (HBV), is also known to have a highly compact genetic organization within its genome, where 4 open reading frames (ORFs) overlap with each other[1–3]. The human HBV belongs to the *Hepadnaviridae* viral family of enveloped and primarily hepatotropic DNA viruses[4]. In the presence of a suitable host this virus is capable of replication and assembly exclusively in hepatocytes where virions are discharged non-cytopathically over the cellular secretory system, as well as sub-viral particles (SVPs). Hepatitis B viral genome has a partially double stranded relaxed-circular (rc) DNA features 4 ORFs: PreS/S, preCore/Core, Pol/RT and X **(Figure 1.1)**[1,2,5,6].

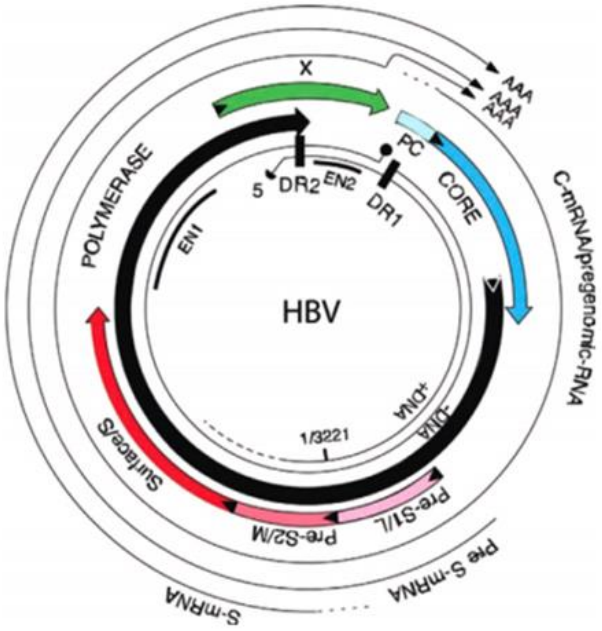

Figure 1.1. HBV genome structure, genes and mRNAs. The 3.2-kb partially double-stranded relaxed circular DNA genome of HBV is displayed in the center. The colored arrows surrounding the HBV rcDNA are representative of the locations of the four overlapping open-reading frames (ORFs) within HBV genome – HBcAg Core (Pre-Core/Core); HBsAg Surface (Pre-S1/L, Pre-S2/M and S); HBV DNA Polymerase and HBx. All open reading frames have a clockwise direction. The outer layer arrows indicate the major HBV mRNAs, all of which end at a common polyadenylation signal located in the core open reading frame. DR1 and DR2 represent 11 base direct repeats that have an important role in viral DNA synthesis. AAA: Polyadenilated Tail; PC: preCore; DR1: Direct repeat 1; DR2: Direct repeat 2. Adapted from *Seeger et al, 2016*.

These ORFs encode for 7-8 known proteins, that are displayed during the viral life cycle of HBV[7]. The preS/S open reading frame encodes for three fundamental but related envelope glycoproteins. These proteins have overlapping ORFs, and synthesis of each one individually is initiated by start codons that are specific for each viral antigen present. They are termed small (S), middle (M) and large (L) proteins. There is another characterization possible, Pre-S, Pre-S2 and Pre-S1 respectively, supported by the fact that surface envelope proteins such as these may be known as Hepatitis B surface antigens (HBsAg, **Figure 1.2**)[2,4,8]. The polymerase/reverse transcriptase (Pol/RT) ORF is considered very complex due to many functions it needs to attend, meaning that this ORF encodes for the viral polymerase, which basically is a multifunctional protein with reverse transcriptase functionalities, as well as DNA-dependent DNA polymerase and RNase H activity[1,2,5]. Additionally, the Pol/RT ORF is also described as functioning as a terminal protein used in priming. The preCore/Core domain encodes for a structural protein of the viral nucleocapsid, the core antigen

(HBcAg)[7] and a non-structural secreted HBV protein, the hepatitis B e antigen (HBeAg)[2,4]. Lastly, the X region encodes a small but regulatory protein (HBx)[9]. This protein is of vital importance for hepatitis B viral replication and regulation of transcription upon infection modulating in concordance host and viral gene expression[10].

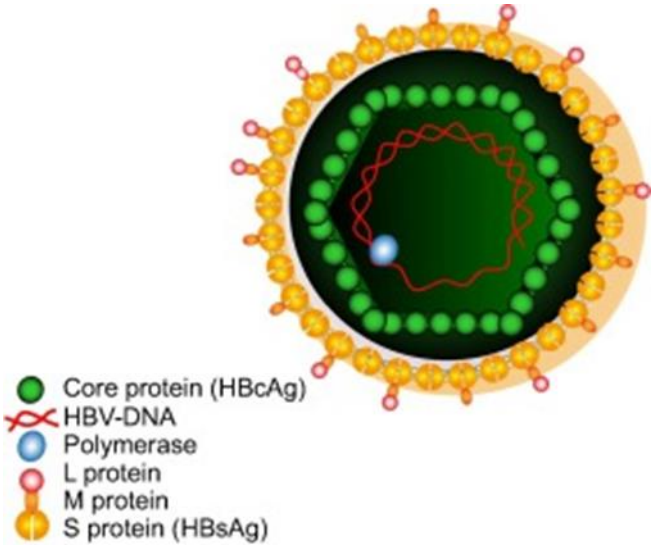

Figure 1.2. HBV virion schematic representation. Structurally, the HBV virion consists of an envelope containing three HBV secreted surface proteins (S-, M-, and L-proteins) and lipids. Also, HBV virion contains an icosahedral nucleocapsid, which is constituted by the core protein (HBcAg) enclosing the viral DNA genome in a covalent linked manner to the terminal protein of the HBV DNA polymerase. Adapted from Pollicino et al, 2014

The life cycle of HBV initiates with the virus attachment to its receptor on the hepatocyte surface **(Figure 1.3)**. Interestingly, the rationale behind this receptor function for HBV remained uncharacterized for years. However, more recently it has been noticed that sodium taurocholate co-transporting polypeptide (NCPT) performs as one possible receptor for the HBV life cycle[8,11]. *In vitro* studies confirmed that the expression of the human NCPT receptor, when placed on HBV non-permissive mouse cell lines, weakened those cells' ability to become permissive. This fact may suggest the requirement of other receptors granting HBV entry into hepatocytes. Another possibility is indeed the lack of host factors that influence viral replication in these animals. Simultaneously, upon hepatocytes viral uptake, the translocation of the HBV nucleocapsid from the cytoplasm to the nucleus occurs. After viral uptake, relaxed circular DNA (rcDNA) is released to the nucleus through nuclear pores and converted into the covalently closed circular DNA (cccDNA). This phenomenon takes place within the nucleoplasm via DNA repair mechanisms of the host himself [1,3,4]. The newly synthesized DNA sequence is then wrapped around histones, so that it may form an episomal chromatinized architecture as mini-chromosomes and responsible for the establishment of chronic HBV[6]. It is essential to point out that cccDNA displays a major function as a stable template for viral replications, as well as a key element for viral persistence. Although this entire pathway is of extreme importance for viral replication, it is used as a transcription template for the 4 poly-A-tailed viral mRNA transcripts and gives insight on some of the features behind Hepatitis B viral infection[1,2,7,8] When transported to the cytoplasm, these mRNAs are translated and give origin to all the different proteins described above, within their ORFs and their place of action[4,12]. Also, is important to mention that this cascade of events might not take place if the nucleocapsid dimerization and self-assembly of the pre-genomic viral RNA (pgRNA), is not reversibly transcribed into rcDNA within the viral capsid. Nevertheless, DNA nucleocapsids within the cytoplasm happen to undergo one of two

resolutions: is recycled into the nucleus to preserve the cccDNA supply levels or it is wrapped and secreted via the endoplasmic reticulum due to their excess amount of proteins produced exceeding the level needed for virion assembly[2]. This excessive number of proteins, mainly envelope proteins, are subjected to dimerization/multimerization processes, which result is a mature growth in the endoplasmic reticulum (ER) and/or Golgi compartments as non-infectious spherical and filamentous SVPs or as virions **(Figure 1.3)**. In a typical manner, these SVPs outnumber the virions values by 1000 to 10000-fold[13–16]. This characteristic may explain why SVPs are constantly found within the circulating immune complexes as well as immune tolerance induction by the mechanism of "viral apoptotic-like mimicry"[13,17]. Like other viruses that affect hepatocytes and the liver, HBV genome integration in the host genome may occur in a randomly manner. Despite not being mandatory for viral replication, viral genome integration is still involved in hepatocyte transformation, as well as a source for new infections[13].

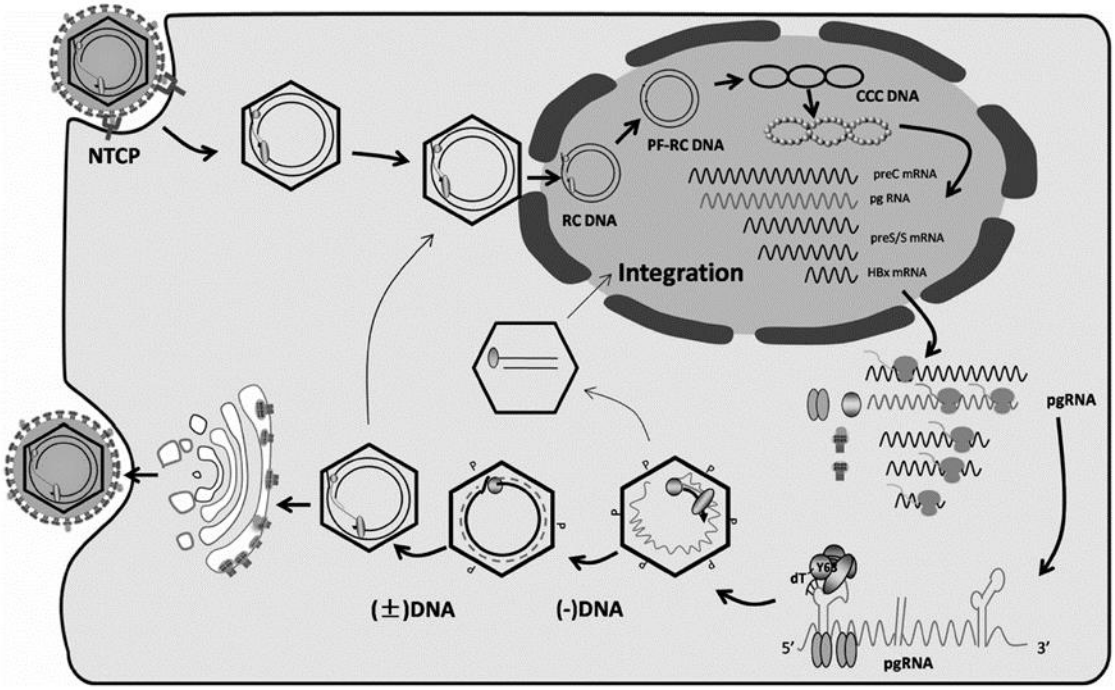

Figure 1.3. HBV life cycle. HBV enters hepatocytes through the NTCP channel, followed by the uncoating and consequent nuclear transport of the rcDNA. The rcDNA is converted in cccDNA, which serves as the template for transcription of the preC RNA, pgRNA, preS/S mRNAs, as well as HBx mRNA. Shortly after these RNAs are transported to cytoplasm, where protein translation takes place. The pgRNA is selectively bundled inside core particles, and subjected to reverse transcription, where it is degradated and gives place to RC DNA. Such mature core particles can be enveloped for release as virions or transported to the nucleus to generate more cccDNA. Adapted from Balmasova et al, 2014.

1.1.1 Genetic Variability of HBV

Due to the lack of reverse transcriptase proofreading activity, there is a movement towards the emergence of new viral mutations affecting HBV genome configuration. As a result, these frequent mutations can induce the appearance of genetically unique viral species, also notated as quasispecies, which will mature due to tension from the different hosts[1]. The relationship between the different characters of an HBV infection, the host, the virus, hepatocytes, immune responses and even antiviral treatments are believed to push towards the emergence of hepatitis B virus mutants. On their

counterpart, these HBV mutants have the competence to evade several immune stimuli or antiviral treatment responses[1,5] These mutations that accumulate in the different viral genomes echo on the duration of active HBV infection and consequently the strength rate of the immune response towards the virus[2]. The relationship between virus/host is always present in the immunologic features of any infectious disease as a measurement for the virus itself survival or dissolution[1,5,18]. Presently, and throughout the analysis of genome-wide nucleotide diversity, 9 genotypes (A-I) and several sub-genotypes were identified for HBV[18]. Also, the existence of a 10th HBV genotype (J) is considered, but its characterization is not fully understood. HBV genotypes differ in their genomic sequences, as well as their geographic distribution around the globe acting in a diverse manner. Nonetheless, all HBV genotypes induce and affect the transmission, progression and pathologies on HBV disease progression. Despite considering that there is not a more common HBV genotype than others, it is thought that HBV genotype C can be considered as critical, since it shows an increased viral mutation rate and high replication capacity, which is correlated to the emergence of cirrhosis and hepatocellular carcinoma (HCC) in chronic HBV patients.

1.2 Immunopathogenesis of Chronic Hepatitis B

Like other infectious diseases, Hepatitis B is known for having two initial stages, an acute and a chronic stage of infection. While these two stages share some similarities, they are still distinct. The main characteristic distinguishing these two stages, besides the timeline of infection, is whether the infection occurred perinatally/early infancy or already in adulthood. The other parameter that differs between the two stages is HBV immunopathogenic mechanisms, with several immune response pathways along the course of disease progression in infected patients.

In acute resolving infections, HBV-infected adults normally acquire self-limited and short-term hepatitis, and it is estimated that in 95% of the infections indeed end with viral clearance, as well as establishment of protective antibodies[19,20]. It is also theorized that innate and adaptive immune responses to HBV are decisive and well timed, when encountering a case of acute resolving infection improving the chance for viral clearance[1]. Thus, it is essential that a strong adaptive T cell reaction takes place early for control of infection, which leads to a cytolytic dependent/independent antiviral outcome via the expression of antiviral cytokines such as interferon γ (IFNγ) and tumor necrosis factor α (TNFα), as well as B-cells stimulation [21,22]. Upon stimulation, B-cells start secreting neutralizing antibodies that alongside antiviral cytokines prevent additional hepatocyte infection and virus spreading[21]. Furthermore, since viral spread is inhibited and hepatocyte turnover mechanisms occur there is a decline in HBV cccDNA levels[1]. However, when the infection progresses to a chronic state HBV specific T-cell function becomes impaired. Therefore, there is a decrease in proliferation, cytotoxic activity and production of antiviral cytokines such as IFNγ and TNFα, leading to phenomenon called T cell exhaustion [23]. During its chronical state, HBV infection spread exerts its effect through various disease stages, which are strongly thought to be associated with age[1]. Interestingly, it was recently observed that HBV infection acquired at younger age, for example perinatally, shows a tendency for evolving to a state of chronic HBV infection. In addition, it is thought to be directly related to their immune profile, since it is less compromised when compared to older infected patients and more susceptible to disease progression[21,22,24].

HBV is acknowledged for its capacity on engaging several immune elements during the natural course of infection, as it manages to adapt and progress through its pathogenesis[25]. Pathologically, HBV chronic patients display a greater risk to develop cirrhosis and HCC. Nonetheless, HBV is unable to have a direct cytopathic outcome on hepatocytes and currently it is well-established that hepatocytes death in chronic hepatitis B (CHB) patients results from their apoptotic state as consequence of immune responses, due to active viral replication and liver

injury[19,26,27]. HBV-specific cytotoxic lymphocytes (CTLs) are considered key players in hepatocytes killing, since they show strong reactivity responses to viral antigens in chronic HBV infection. Per contra, these lymphocytes are not strong enough to mediate the full eradication of HBV[19,28,29]. In concordance, CTLs try to mobilize HBV-nonspecific inflammatory cells such $CD8^+$ T cells, natural killer (NK) cells and neutrophils[19,30]. All these immunological players consequently induce CHB immunopathological functions. Despite all the continuous immune responses against HBV infection, it is still not clear why viral clearance is so rare in HBV patients, since HBV-specific T cells are exhausted and incapable of killing infected hepatocytes, which means no liver injury and no control of infection[19,25,31]. During HBV chronic infection, there is a progressive synergistic relation between the viral replication and host immune responses to HBV[1]. However, not all patients exhibiting chronic HBV infection progress and develop CHB[1]. CHB has been divided in different stages according to its natural viral progression [25,32]. Also, its nomenclature has changed over time, since new scientific breakthroughs about the disease were discovered, as well as parameters present in HBV regulation. Recently, chronic HBV infection is divided into 4/5 branches or states, depending on the report[1]. Chronic HBV nomenclature bears in mind distinct parameters such as the presence of HBeAg (HB e antigen), HBV DNA levels, alanine aminotransferase (ALT) values and the presence/absence of liver inflammation[25,31] (**Figure 1.4**). Also, the new chronic states annotations are mainly based in two central attributes of chronicity: infection and hepatitis[1]. Nonetheless, it is wrong to establish an immediate viral infection stage classification, since a single measurement of HBV replication markers and disease activity mediators might not be enough to take solid conclusions. Thus, evaluation and monitoring of the different disease parameters is necessary to speculate about possible therapeutic approaches administered individually or in group against HBV. Curiously, despite the 4/5 existing phases for HBV chronic infection, it does not necessarily mean that all phases follow a sequential order.

CHB infection initial stage is nominated HBeAg-positive or, as previously called, "immune tolerant phase"[1,4] (**Figure 1.4**). Also, this stage is well-characterized by the presence of HBeAg in patient's serum, high viral load (HBV DNA) and ALT values within the documented range of approximately 40 IU/L[1,4,25,31,33,34]. As for the liver inflammatory level within this infection stage, there is no apparent necroinflammation or fibrosis in liver tissues[1,25,34,35]. However, HBV DNA integration, as well as clonal hepatocyte spread, might still occur. Furthermore, this could be an indication of initial hepatocarcinogenesis manifestations[1,4,36,37]. The preservation of HBV specific T-cell functionalities is more frequent and prolonged in this stage, progressing until young adulthood if patients are infected perinatally[4,36–38]. Importantly, loss and seroconversion of HBeAg to anti-HBeAg is normally low in this phase, which decreases the chances of disease remission[4,25,39,40].

As HBV infection progresses, ALT values show a tendency to increase leading to the next chronic HBV stage - CHB HBeAg positive or "immune clearance stage"[1,4] (**Figure 1.4**). Nonetheless, HBeAg frequency in patients' serum and HBV DNA levels remain similar to the previous stage, whereas in the liver the signs of necroinflammation and fibrosis become moderate or even severe in singular cases[1,4,38,41]. This medical condition is frequently found among subjects infected during adulthood and it may arise after numerous years of the previous disease state[1]. CHB patients in this stage can have one of two outcomes – manage to seroconvert HBeAg and suppress HBV DNA levels or fail to control HBV infection – the latter allowing the entrance in the HBeAg negative CHB phase [1,4,38,42].

Following this, since HBeAg loss gives rise to the production of anti-HBeAg, CHB patients eventually reach the "inactive carrier stage" or currently noted as HBeAg negative CHB infection phase[1,4,25,43] (**Figure 1.4**). At this stage, HBV patients are subjected to a change in the parameters

defining chronic HBV infection[4]. Patients' serum shows traces of anti-HBeAg in constant rise, HBV DNA levels are considered minimal and ALT parameter is described as stable and in mild values. In addition, the risk for disease progression into a cirrhosis and HCC in this stage is considered low [1,38,44]. Although this CHB phase is considered stable, there are cases of patients suddenly progressing deeper into the infection, which could be related to the emergence in viral load with no apparent changes in liver ALT values, but residual signs of necroinflammation[45]. The HBeAg negative CHB infection phase is also characterized for showing low levels of HBsAg loss, which means that seroconversion of HBV's antigen is minimal and is extremely difficult to spot on in patient's serum [1,45]. Nonetheless, in 20%-30% of the inactive HBsAg carriers, spontaneous reactivation of hepatitis B may occur, which can lead to a disease progression and development of liver damage and decompensation [4]. As HBV infection progresses, patients are likely to enter the HBeAg-negative CHB stage. Here, the lack of HBeAg in patients' serum is characteristic, as well as fluctuations in HBV DNA levels, which might be correlated to an increase of ALT in the liver. The aforementioned phase is associated with low rates of spontaneous disease remission, perhaps since nearly all the patients within this phase demonstrate to have HBV variants accommodated within the precure/basal core promoter regions, leading to an impairment/abolishment of HBeAg manifestation.

The last official phase in chronic HBV infection is called the HBsAg-negative phase but can also be denominated by "occult HBV infection"[1] (**Figure 1.4**). The latter denomination relies on the fact that this stage is hypothesized to occur during all the previous HBV infection phases mentioned, though its diagnostic time point is not fully understood. Furthermore, this stage is characterized for showing no detectable antibodies for HBsAg in patients' serum, positive antibodies for HBcAg (anti-HBcAg) and normal ALT levels. Also, the majority of patients in this late HBV infection stage show undetectable traces of cccDNA) in the serum, although it is normally detected upon liver histological exams. HBsAg loss and seroconversion in this stage is correlated to disease progression into more extreme pathologies such as cirrhosis or HCC, since this HBV antigen is known to mediate HBV progression. Nonetheless, CHB patients' survival rate is increased if HBsAg loss takes place before liver flares and cirrhosis outbreaks. However, in cases where patients show signs of cirrhosis or HCC but HBsAg loss is insignificant, it is possible that HBV reactivates, leading to a possible death.

Table 1.1 Phases of chronic HBV infection.

Marker	HBeAg-positive infection	HBeAg-positive hepatitis	HBeAg-negative infection	HBeAg-negative hepatitis	Resolved infection
HBsAg	High	Intermediate	Low	Intermediate	Negative
HBeAg	Positive	Positive	Negative	Negative	Negative
HBV DNA	Very high	High	<2000-20 000 IU/ mL	>2000-20 000 IU/ mL	Undetectable
ALT	Normal	Increased	Normal	Increased	Normal
Risk for disease progression	Minimal	Yes	Minimal	Yes	None
Treatment	Monitor	NA/PEG-IFN	Monitor	NA/PEG-IFN	None[a]
LHBs			Low	High	
Anti-HBc	Low	High	Low	High	Positive
HBcrAg	Very high	High	Intermediate	Low	Undetectable
HBV RNA	Very high	High	High-intermediate	Intermediate-low	

Legend: ALT, alaninaminotransferase; IU, international units; NA, nucleos(t)ide analogues; PEG-IFN, pegylated interferon alfa; HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; LHBs, large hepatitis B surface protein; anti-HBc, antibodies to hepatitis B core antigen; HBcrAg, hepatitis B core-related antigen; RNA, ribonucleic acid; DNA, deoxyribonucleic acid.The risk for disease progression and the need for treatment is indicated by colour shading: red: high risk for disease progression / treatment required, yellow: low risk for disease progression / monitoring required, green: no disease progression / no action required. aMonitor in case of cirrhosis or immunosuppression.

1.2.1 T-Cell exhaustion

Innate and adaptive immunocyte activation is regulated by a set of inhibitory surface receptor-ligand pairs or checkpoint inhibitors[46]. It is well established that the presence of anti-viral T cells contributes to the eradication of acute infections, restrains the resurgence of latent viral infections and controls the viral loads during chronic infection, as these cells are crucial elements of the adaptive immune response[47]. Typically, naive T cells recognize antigen peptides presented via their T-cell receptor (TCR) and become activated, being liable to clonal expansion as well as differentiation into potent effectors[48]. When in a differentiated effector state, T cells show high cytotoxic levels, produce effector cytokines such as IFNγ and TNFα and express chemokine and homing receptors, which allows them to migrate to peripheral tissues and infection sites[48–50]. However, the host immune system fails to respond if there is a continuous TCR stimulation due to the persistence of high levels of antigens, leading T cells to enter a state nominated T cell exhaustion[51,52]. Fundamentally, T cell exhaustion is characterized by the endurance of high levels of antigens, that leads to a repeated T cell dysfunctional state and an increase of distinct inhibitor receptors (IR), a gradual depletion of effector cell functions translated by the loss of effector cytokine secretion levels (IL-2, IFNγ and TNFα), as well as a change of these cells metabolism and transcriptional profile [51,53–55]. Also it is well-established that exhausted T cells are characterized by the upregulation of surface markers such as PD-1, TIM3 and LAG3, investigated during the course of this project.

1.3 Treatment Strategies

Hepatitis B virus (HBV) remains a major healthcare challenge, and according to the World Health Organization (WHO), hepatitis viral infection is still the eight most frequent cause of mortality worldwide[56,57]). HBV is estimated to affect approximately 400 million people around the globe, and despite the existence of a prophylactic vaccine, there is still no definitive curative treatment for the more than 240 million people globally that are considered chronically infected with HBV[57]. CHB patients show a higher risk of death due to an inherent tendency to develop other severe pathologies, including liver cirrhosis and HCC[8,57] . Prophylactic vaccination against HBV contains the envelope protein S and is either produced by processing of HBsAg retrieved from HBV carriers' plasma, or from the yeast specie *Saccharomyces cerevisiae*, holding a recombinant DNA plasmid that also expresses the S protein[58–60]. Currently, there is another type of prophylactic vaccine, which encompasses the S and M envelope proteins obtained from genetically engineered ovary cell lines[58–60]. Even though HBV infection can be prevented by a recombinant yeast-derived prophylactic vaccine, the treatment is mostly available in developed countries and is mainly administered to infants and newborns. It is still extremely difficult for some undeveloped countries individuals to be granted access and acquire this preventive treatment, mainly due to its limited availability and high cost. Nonetheless, it appears that 184 of the 196 countries in the world are able to get a safe and effective prophylactic vaccine, at least for children[8]). In compliance with the number of chronically infected HBV patients globally and high mortality rate, the formulation of new and more efficient therapies is necessary. Also, efforts are being made by the scientific community to develop different therapeutic vaccines to substitute the prophylactic treatment, which can endure the clearance of circulating HBsAg and convert these to a state of recognition by antibodies of HBsAg, also called HBsAg serocoversion[28,57].

1.3.1 Nucleos(t)ide analogues and Interferon-Based therapies

Currently, there are two treatment therapies approved for CHB patients: nucleos(t)ide analogues (NUCs) and IFN-based therapies such as pegylated interferon-α (PEG-IFNα)[56,57]. Despite demonstrating very interesting results among hepatitis B infected patients, unfortunately these

treatment regiments are still far from a satisfactory cure and complete eradication of the virus. When used separately, these two therapies work in distinctive manners. NUCs therapy impact positively on adaptive immunity[61], suppressing viral replication by directly targeting virion synthesis[61,62]. However, this therapeutic drug does not successfully eliminate the nuclear persistence form of the virus genome, cccDNA, from all liver infected hepatocytes[56,57]. This eventually leads to an HBV infection rebound and hepatic flares upon treatment withdrawal[56,63,64]. For chronically infected HBV patients, NUCs treatment is considered life-long, representing an immense financial liability and latent systemic drug toxicity[56,57,63]. Another concern about using NUCs as a therapy against HBV infection is the possible appearance of drug resistance mutants in chronic patients due to its prolonged use[65]. Thus, these drugs can also be nominated as antiviral inhibitors, acting on HBV reverse transcriptase activity and disturbing its viral life cycle[66]. There are some NUCs available for clinical treatment, such as lamivudine (LAM), adefovir, entecavir (ETV), telbivudine and tenofovir (TDF)[8,67]. Although the use of NUCs show some contradictory effects, this antiviral therapy has shown to be effective as a functional cure. This kind of treatment provides the normalization in ALT levels, suppression of HBV DNA in patients, histological improvements and decreased liver inflammation and fibrosis[8]. As for HBeAg and HBsAg, it is hypothesized that NUCs treatment leads to a possible loss and seroconversion of both HBV specific antigens, but more studies should be performed in the clinics[8].

Besides using NUCs as a controlled treatment against CHB infections, it is currently very common to combine this medication with an IFN-based therapy[57]. IFN-based therapies, also called cytokine-mediated therapies, are new treatment strategies known for the enhancement of the immune system through the production of interferon-stimulated genes (ISGs) and their cytotoxic activity in killing HBV infected cells[66,68]. This therapeutic approach includes PEG-IFNα, and together with NUCs is the best line of attack against HBV infection, with particular attention for CHB patients[66]. In contrast to NUCs antiviral treatment, PEG-IFNα therapies currently show a higher efficacy response on the innate immune response[61,69,70]. Despite this, PEG-IFNα treatment and other IFN-based therapies can only grant an HBV cure to 10-15% of the treated patients[57]. Nonetheless, this treatment is strongly recommended for chronically infected HBV patients with no medical history of liver diseases, since PEG-IFNα is associated with severe side effects, including flu-like symptoms and the occurrence of hepatic flares[57,66,71,72]. Interferon α (IFNα) drugs are typically administered subcutaneously/intramuscularly on CHB patients, and lead to a decline of HBV DNA serum levels and viral replication rate. Consequently, this causes a reduction in HBV transcription, as well as enhancer activity in the host genome[65,66,73]. Also, IFNα therapy results in a decay of HBV virions, DNA polymerase, as well as HBcAg levels linked to HBV virions due to an inhibition of its reverse transcriptase activity[65,66,74]. The use of PEG-IFNα compared to standard IFNα has resulted in a significant reduction of HBV DNA and ALT levels in CHB[66,75]. Interestingly, IFNα was recognized as an up-regulator of APOBEC3A/C3B proteins, causing cystidine deanimation and degradation of cccDNA, without affecting the host genome[3,66,76]. These two drug treatment strategies are often combined, demonstrating higher reduction in HBV DNA serum levels, inhibition of HBV replication and lower ALT levels. However, even when combined, these treatments are unable to eradicate cccDNA from all infected cells, and it is very common for patients to experience a rebound of the viral load and ALT levels increase upon withdrawal of the medication. This might be due to the increase of cccDNA persistence as main cause and drug-resistance to the medication, hence it is considered a life-long treatment[61,65,66].

1.3.2 Therapeutic Vaccination

Since HBV is capable of hampering adaptive and innate immune responses, it is of the utmost importance to find a new therapeutic approach that boosts the immune system and further enhances

the efficacy of the current antiviral therapeutic regimen[58]. The development of therapeutic vaccination is meant to activate the immune system in infected patients, in order to fight against, control, or ideally eliminate the established infectious pathogen[57]. In CHB patients, the objective is to reinforce the immune system to a point in which cccDNA clearance from HBV infected hepatocytes is achieved. Many studies have been published on the development of therapeutic vaccination against the HBV[58]. For the past 20 years, several clinical trials have been made regarding numerous therapeutic vaccination strategies, but the end results are far from excellent and cccDNA clearance is still impossible to accomplish[57]. Nonetheless, the scientific community is working to develop a therapeutic vaccine that can grant a definitive cure for CHB patients, instead of a functional one obtained with the available antiviral treatments. To the best of our knowledge, recombinant peptide-based vaccines[77–85], DNA-based vaccines[86–88], viral vector-based vaccines[89,90] and cell-based vaccines [91,92] are being studied and are presently the most promising therapeutic vaccination approach[57,58].

In a simplified manner, the primary attempts to develop a therapeutic vaccine for HBV were peptide-based vaccines, and Pol S and co-workers were the first conducting a HBV therapeutic vaccination trial to test the efficacy of this vaccine nature in CHB patients[58,77]. This kind of therapeutic vaccination is easily produced and induces high titers of HBV-specific antibodies. Despite the efforts, peptide-based vaccines still show weak cellular immunity responses and require adjuvant and continuous administrations[58,93]. However, it seems that there is a substantial decrease in serum HBV DNA levels and viral replication, but no significant diminished in HBsAg levels in most of the patients tested[58,77]. Still, there is evidence that peptide-based vaccines for HBV will activate T cells responses, reduce HBV viral load and quicken HBeAg seroconversion frequency in CHB patients[58,94]. Another peptide-based vaccine approach involves the combination of recombinant HBV core particles (HBcAg) with HBsAg[58,95]. The combination of recombinant HBsAg with anti-HBs antibodies to form an antigen-antibody immune complex (IC) became a motivating method for developing therapeutic HBV vaccination[58]. Noteworthy, it is thought that these ICs may improve the chance of HBsAg being captured by antigen presenting cells (APCs), therefore enhancing the immunogenic effects of the vaccine and inducing more potent HBs-specific T cell responses[58,96]. All peptide based vaccines so far have failed to induce sustained HBV-specific T cell responses in CHB patients. Though, with all the clinical trials performed so far, the medical community suggests that repetitive adjuvant vaccination might result in an inflammatory environment and stimulate pre-existing T cells in the liver[58,81]. This hypothesis is based on T cell responses detected upon therapeutic vaccination due to an effect registered on pre-existing T cells, indicating that patients with active liver inflammation may benefit more from therapeutic vaccination[58,81,82].

On the other hand, DNA-based vaccines encoding HBV envelope proteins are also under investigation as a therapeutic vaccination against HBV chronically infected patients[57,97,98]. This vaccination type induces humoral and cellular immune responses, including $CD8^+$ and $CD4^+$ T cell responses[58,99]. It seems that patients' immune response after DNA-based vaccines administration is similar to that of individuals whose HBV infection was cleared. Furthermore, since HBV-specific IFN-γ-secreting T cells show upregulated levels upon treatment with this vaccination regimen, this strategy is promising, since it induces effective but transient T cell responses[58,88,99,100]. However, DNA-based vaccines fail to achieve HBeAg and HBsAg seroconversion, and they are not able to induce a consistent T cell response with reduction of viremia levels in chronic HBV patients[57,97,98].

Viral vector-based vaccines are another therapeutic vaccination approach to cure HBV, as these vaccines foundation is based on live attenuated viruses capable of broad stimulation and sustained immune responses[58]. In particular, adenoviral and modified vaccinia virus *Ankara* (MVA)-based vectors have been tested in clinical trials, since these vaccines have an extensive stimulation

range in immune responses like T cell-mediated immunity[57]. Nevertheless, vaccination strategies should combine different viral vector-based vaccines with other vaccination therapies like DNA-based vaccines, and recombinant antigens[57,90]. Finally, a vaccine strategy called cell-based vaccination was developed based on the role of innate immunity in HBV infection, as it aims to stimulate pattern recognition receptors (PRR). The objective is to induce a stimulatory response on different immune regulators like toll-like receptors (TLRs) and APC receptors (e.g. dendritic cell, or DC, receptors), which in turn can stimulate specific $CD4^+$ and $CD8^+$ T cells and direct them at HBV-specific infected cells[58,101].

New and more compelling therapeutic vaccination strategies have been developed and entering clinical trials recently (**Table 1.2**). However, these likely require new potent vaccine components and schemes that could induce more effective humoral and cellular immune responses. Besides this, it is necessary to include checkpoint inhibitors or other similar strategies that could overcome T cell exhaustion in chronic HBV infected patients, as well as lowering HBV specific antigen levels as a preventive action for T cell attrition in patients with low antigen loads[57].

Table 1.2. New therapeutic strategies against chronic HBV infection. These strategies aim to target either a viral target, or modulate immune system components to overcome the viral infection. Adapted from Gill, Upkar S and Kennedy, Patrick T.F, 201.

Viral Targets	Entry Inhibitors	Interaction with sodium (Na+) - Taurocholate Cotransporting Polypeptide (NTCP) and decreases viral infection rate i.e Myrcludex
	Silencing & Eliminating cccDNA	cccDNA targeting through use of antiviral cytokines, blockade of RcDNA or via epigenetic regulation possibly leads to viral DNA degradation and elimination from infected hepatocytes i.e CRISPR/Cas9 and histone deacetylase (HDAC) inhibitors
	Secretion Inhibitors	Inhibition of HBsAg secretion i.e nucleic acid polymers
	HBV Polymerase Inhibitors	Promote the inhibition of the reverse transcriptase mechanism by heapatitis B polymerase and help in suppressing viral expansion
	Core Allosteric Modulators (CpAM)	These agents stimulate the inhibition of nucleocapsid assembly, during hepatitis B life cycle, which incapacitates pgRNA encapsidation and results in the capture of viral RcDNA
	Silencing RNA	Prevent HBV replication by using HBV-specific small molecules that interfere and silence RNA resulting in reduced cccDNA levels in infected hepatocytes i.e RNA interference (RNAi)
Immune Targets	Immune Stimulation	Induce a stimulatory effect in the immune system. Agents for immune stimulation include pattern recognition receptor (PRR) agonists, Toll-like receptor 7 (TLR7, GS-9620), as well as TLR1/2, retinoic acid inducible gene I (RIG-I), and stimulator of interferon genes (STING)
	Immune Modulation & Cytokines	Innate cells have been identified for their role in immune tolerance. For example, NK cells demonstrate to have a regulatory role upon HBV-specific T cells via upregulation of a death receptor and myeloid derived suppressor cells have been shown to influence the adaptive immune response. On the other hand, the modulation of innate-adaptive interactions could also hold therapeutic promise, as cytokines such as TNFα, IL-2 and IL-12 have been implicated in HBV replication inhibition in vitro.
	Checkpoint Inhibitors	HBV-specific T cell are exhausted and overexpress inhibitory molecules such as programmed death-ligand 1 (PD-1), TIM3 and LAG3. However, in case of these molecules blockade as shown potential in vitro, with special attention in hepatocellular carcinoma medical conditions.
	T cell Therapies	Increasing the number of HBV-specific T cells by autologous infusion of T cells expressing chimeric antigen receptors (CARs) or by engineering T cells to overexpress human leukocyte antigen (HLA)-restricted HBV-specific T cell receptors (TCRs).

1.4 Mucosal-associated Invariant T Cells: What are they?

From an immunological point of view, the liver is considered one of the most important organs in the human body[102]. This aspect is supported by the fact that, even after being continuously exposed to non-self-food and microbial-derived products from the gut, the liver remains sterile and tolerogenic in homeostasis[103]. Nonetheless, in situations where the intestinal mucosal defenses are

ruptured, or in presence of a systemic infection, this organ acts as a second 'firewall', through its enrichment with innate effector cells, which rapidly respond to infections or tissue damage[102,103].

Mucosal-associated invariant T (MAIT) cells are described as the most abundant subset of unconventional and innate-like T cells in humans[104]. MAIT cells are evolutionary conserved among different species and seem to be highly abundant in human mucosal tissues, peripheral blood and liver[104]. Also, there are evidence suggesting that MAIT cell can recognize microbial riboflavin metabolites (Vitamin B_2) secreted by numerous bacteria and fungi species presented via the non-polymorphic major-histocompatibility complex (MHC) Class-I related molecule (MR1)[105,106]. Due to the strong evolutionary conservation of this molecule (MR1) and the lack of riboflavin metabolite antigens variety, it is proposed that these cells are homogenous in their responses against several microbes that hold the biosynthesis pathway for riboflavin metabolites[104–106]. These T cells subset are defined by the expression of a semi-invariant αβ TCR) combined with a specific segment (Vα7.2) and high expression levels of the C-type lectin receptor CD161. These two receptors are well-established for describing, define and isolate MAIT cells in healthy humans. However, it seems that recent studies showed that the IL-18 receptor α subunit (IL-18Rα), in combination with the TCRVα7.2 marker, can be used to define MAIT cells in humans[107–109]. It is known that MAIT cells demonstrate higher tendency to accumulate at the infection sites, providing a protective role in some experimental infection models[104].

1.4.1 MAIT cells Development and Tissue Distribution

In resemblance to other T cells subset like NK T cells, MAIT cells development starts in the thymus[110,111], where these cells undergo a pre-selection by MR1-expressing $CD8^+CD4^+$ thymocytes[112]. Using transgenic mouse models and thymic organ cultures, Seach and colleagues were able to determine that MR1-expressing double positive thymocytes have a vital role in MAIT cells selection mechanisms during their maturation development[113,114]. Also, other cells types such as thymic B cells and DCs are apparently redundant in MAIT cells selection in the thymus[112]. MAIT cells phenotype shows differences, depending on these cells' location in the human body, as well as their developmental stage. When in the thymus and cord blood, MAIT cells exhibit a naive phenotype, characterized by being $CD45RA^+CD45RO^-$ [111,115,116]. On the other hand, peripheral blood MAIT cells are $CD45RA^-CD45RO^+CD28^+CCR7^-CD62L^-$, which marks these cells as effector memory cells[110,111,115]. Nevertheless, Koay and colleagues proposed that MAIT cells maturation and development consists on a three-stage pathway, and is based on the expression of two markers in the thymus, CD161 and CD27 (**Figure 1.5**) [117].

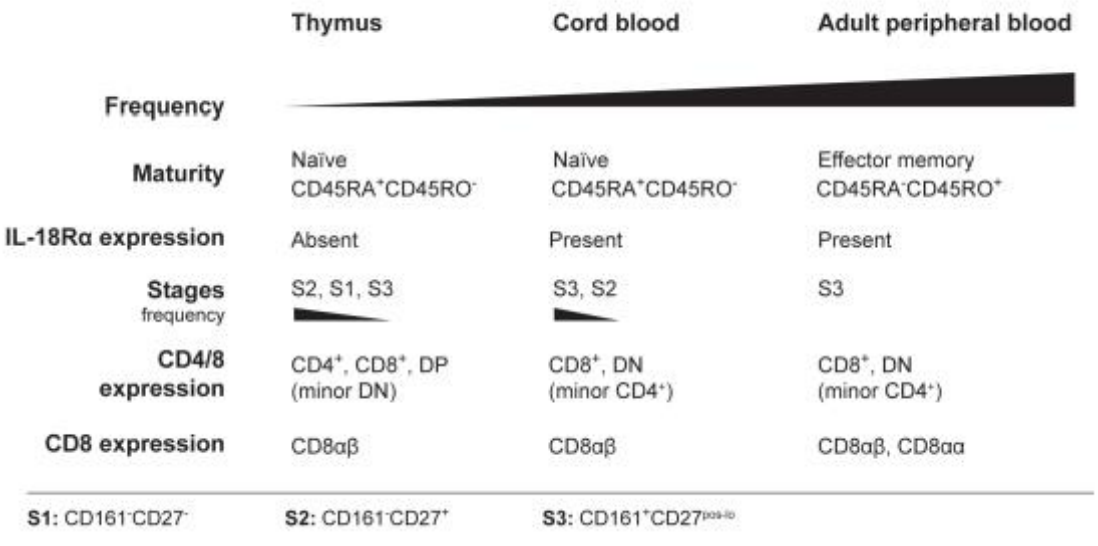

Figure 1.4 MAIT cells developmental stages, phenotype and frequency in the thymus, cord and adult peripheral blood. Adapted from Dias Joana, 2017.

In stage 1, in thymus, $CD161^{-}CD27^{-}$ MAIT cells would be predominantly $CD8^{+}CD4^{+}$ or $CD4^{+}$ only, whereas in stage 2, besides the latter described, MAIT cells could also be $CD8^{+}$ only. Nonetheless, in stage 2 MAIT cells could be defined for being $CD161^{-}CD27^{+}$, characterizing this development stage in the thymus. Lastly, during stage 3, MAIT cells are $CD161^{+}CD27^{+}$, in the thymus as well as mostly $CD8^{+}$ and $CD8^{-}CD4^{-}$ in the thymus. However, MAIT cells in the latter stage are rare in the thymus, while abundant of cord and peripheral blood, which suggests that MAIT cell functional maturation occurs after these cell are translocated out of the thymus[117]. Besides this, MAIT cells are known for expressing the CD8αβ co-receptor exclusively in the thymus and cord blood[116,117], while adult peripheral blood MAIT cells show evidence of CD8αβ or CD8αα expression (Figure 1.5)[111,116,117]. Due to their effector memory phenotype, human peripheral blood MAIT cells are referenced as expressing different transcriptional factors such as PLZF, RORγt[115,118,119], as well as T-bet, Helios, and Eomes at low, intermediate and high levels, respectively[118]. These transcription factors are known for having a role in effector function, proliferation and activation in $CD8^{+}$ T cells[120–122]. Furthermore, adult peripheral blood MAIT cells also express receptors for IL-12, IL-23, and IL-7 [115,123,124], adding up to IL-18R, a well-established MAIT cell receptor. The NK cell receptor NKG2D, the T cell activation antigen CD26, and the multidrug resistance protein 1 (MDR1) are also described as being expressed by MAIT cells at different degrees[115,125]. It is strongly suggested that peripheral blood MAIT cell population tends to gradually expand with age[126–128] and reaches a frequency ten times larger than initially seen in the thymus and cord blood[117]. MAIT cell populations range between 1 and 10% of the total circulating T cells[111,115], although their frequency is associated to a high variability among individuals. Also, the frequencies of $CD8^{+}$ and $CD4^{+}$ MAIT cells show a tendency to inversely decrease and increase with age, respectively[127,128].

Adult peripheral blood MAIT cells are known to express a very distinct mixture of chemokine receptors which moderate their cellular trafficking to the peripheral tissues[102]. Indeed, MAIT cells prefer to home to the peripheral tissues, mainly due to the expression of CCR6 and CXCR6, liver-homing receptors, as well as gut-homing integrin αβ47 and intermediate levels of CCR9, which is also expressed by T cells[102,115,129]. These receptors have an interesting function in lymphocyte migration to the gut. Human MAIT cells percentage is enhanced in the liver, constituting approximately 15% to 50% of the hepatic T cells, staging as the most predominant T cell subset population expressing the markers CD161 and CD56[115,123,130–132]. Despite being considered the largest T cell subset and preferably allocated within the gut, MAIT cells present highly variable frequencies, according to which peripheral tissue they are encountered in[102,104,132]. MAIT cells have been found in several compartments of the small intestine, ileum, duodenum and jejunum, as well as within the large intestine, colon and rectum[102]. MAIT cells are also abundant in peripheral blood (≈1-10% of T cells) in which they are representative of a homogenous subset of $CD4^{-}$ T cells known to express the TRAV1-2 chain together with the CD161, CD26 or IL-18Rα markers [104,111,115,116]. The presence and expression of chemokine receptors, such as CXCR6 and CCR5, illustrates these cells ability to transit to the lungs (≈2-4% of T cells)[115,132]. There is also evidence of the presence of MAIT cells in the stomach (≈2.5% of T cells). More recently it was discovered that MAIT cells have a small frequency in the endometrium and cervix (≈1-2% of T cells), and partially in the human skin. Transcripts for MAIT cells T cell receptor were also detected in other human organs, including kidneys, the ovaries and prostate [133]. Although MAIT cells are widely distributed among the human tissues, studies show traces of these cells in the lymphoid tissues, but in a low frequency[115]. This aspect may be related to the lack of expression of CCR7 and CD62L, two receptors known to have an important function in lymph node homing[132,134,135]. Since MAIT cells are widely distributed within the human body and are highly frequent in the liver, these cells might be a new approach for HBV, as well as other liver

diseases as a possible new therapeutic approach, since MAIT cells can sustain exhaustion and show activation in adverse environments.

1.4.2 Antigen presentation to MAIT cells

1.4.2.1 - MAIT Cell TCR

In 1993, while evaluating $CD4^{-}CD8^{-}$ human T cells TCR repertoire, Porcelli uncovered the presence of two invariant TCRα chains sequences - Vα24-Jα18 and Vα7.2-Jα33[136]. These TCRα chains were characterized based on one Vα gene coupled to one Jα within the CDR3α loop region in the TCR and are currently notated as TRAV10 and TRAV1, respectively [106,137,138]. Later, these invariant TCR sequences were shown to be characteristic of NK T cells and MAIT cells, two innate-like subsets of canonical T cells[110,139]. Nowadays, it is well-established that, besides being relatively evolutionary conserved among mammals, MAIT cells' TCR is also considered semi-invariant, which is strongly related to MAIT cells attributes regarding the adaptive and innate immune system[104,108,113,140]. Furthermore, the majority of human MAIT cells display the canonical TCRα chain defined by a Vα7.2-Jα33 rearrangement[110,133,141]. However, it is now known that a minority of these cells TCRα chain can represented by different rearrangements - Vα7.2-Jα12 or Vα7.2-Jα20 [102,132,133,141]. Human MAIT cells TCRα preferentially pair-wise with TCRβ-chains Vβ2 or Vβ13.2, indicating lack of TCR diversity[113,133]. Nonetheless, this MAIT cells feature can be seen as advantageous in recognition and response initiation upon encounters with different pathogens[113,114].

1.4.2.2 – MR1

MR1 is a non-polymorphic gene located on chromosome 1 in humans and was first sequenced in 1995[102,132,142]. Currently, this gene is well-established as an antigen-presenting molecule and is ubiquitously expressed in all cells[138,140]. Nonetheless, in a steady state, endogenous surface MR1 expression has been difficult to detect with anti-MR1 staining on non-transfected MR1 cells[108,132,140,143–146]. Interestingly, the MR1 molecule is highly conserved among mammals, displaying an increased homology with classical MHC class I molecules in humans and mice regarding their α1 and α2 domains [106,132,140,142,147,148]. So far and through alternative splicing, four human MR1 isoforms have been characterized and are notated as MR1A to MR1D[106,132,147]. The A isoform resembles the full-length protein originally discovered with 341 amino acids and all the structural characteristics of the classical MHC class I molecules[106,132]. These features include a leader sequence or signal peptide, three extracellular domains (α1 and α2 domains which form the ligand-binding pocket containing conserved residues of MHC-I molecules and the α3 domain that interacts with riboflavin residues), a transmembrane domain and a short intracytoplasmic domain with unknown trafficking function so far[106,132,149–151]. As for the remaining human MR1 isoforms all seem to be non-functional, since they lack some of the structural domains preserved in the full-length protein[106,152]. However, the MR1B isoform despite lacking the α3 domain it can still function as an antigen-presenting molecule due to the conservation of the transmembrane domain[106,153]. This isoform is expressed on the cell surface forming homodimers and consequently activating MAIT cells in the presence of bacterial species, despite absence of β2m (riboflavin)[153,154]. *In vitro* studies performed on MAIT cells activation and MR1 blockade have given insight on MR1 and suggested that MR1 antigen presentation is β2m-dependent but TAP-independent[110,114,146]. Currently, it is widely accepted that MR1 presents lipid-based and vitamin B-based antigens[143]. This observation was proved in crystallography studies where MAIT cells exhibited reactivity in the presence of bacterial species that could synthesize riboflavin (Vitamin B_2) or similar precursors due to MR1[105,114,143,149]. Vitamin B_2 metabolites would bind to MR1 an activate MAIT cells [105,149]. Structurally, MR1 and MHC-I are similar both forming a heterodimer

with B_2m and with α1 and α2 domains having a two α-helices sitting atop a 7-strand antiparallel β-sheet rearrangement[143,149]. Furthermore, the MR1 structural groove consists of two distinctive sections – A'pocket and F'pocket[143]. There is a direct alignment of the A´pocket with aromatic and polar residues, which potentiates the binding of pyrimidine, lumazine and pterin-based antigens[143]. Upon bacterial infections the capacity of MR1 to sequester the antigens above mentioned is increased due to the presence of a Lys residue (K43) at the base of the A'pocket and forming a Schiff-base (covalent bond) with the carbonyl group of the antigen itself[143]. In a curious manner, this covalent bond is characterized as common feature of MR1. In opposition to the A'pocket, the F'pocket in the MR1 groove is much trivial and appears to be perfectly lined with primarily polar residues[143,149,155,156]. To the best of our knowledge, there are not physiological ligands characterized yet and the significance of this region is not fully understood. Despite the structural similarities between MR1 and MHC-I molecules, MR1 trafficking is way distinct and its intracellular location is still a debatable subject among the scientific community[132,143]. Presently, it is strongly suggested that most of the pre-synthesized MR1 is confined in the ER, in the absence of bound ligands and adopting an incompletely folded state, meaning no β_2m association[132,140,143,146,157]. However, there are reports suggesting that MR1 besides the ER is also present in late endosomes and lysosomes which facilitates association with β_2m[146,158]. Nonetheless, it is known that soluble ligands are favourite in binding to MR1 when in the ER[157]. The increment in ligand availability and the presence of a Lys residue (K43), previously referred, prompts MR1 to become completely folded due to association with β_2m covalently bounded[132,140,157]. After the MR1-β_2m-ligand is created, MR1 cell surface expression levels increase[132,140,157–159]. This complex is internalized and degraded via endocytosis in late endosomes and lysosomes, where only 5% of the total MR1 present is recycled to cell surface and promoting new ligand exchange actions[132,143,157,158]. All this process is in concordance to what is known about fixed APCs like MR1 and their role in MAIT cells activation, when in presence of ligand-producing microbes[108,132,160]. Still, there are a few questions answered regarding MR1 and its antigen presentation role in MAIT cells[108,160]. Also, it is still unclear in which way ligands reach the ER and why several ligands are incapable to induce MAIT cell activation effectively despite binding to MR1[114].

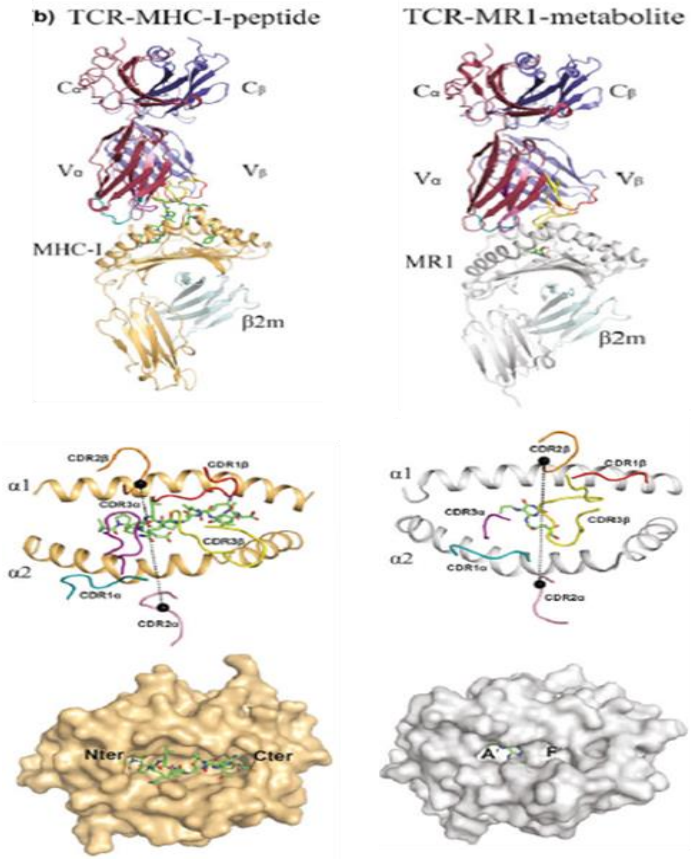

Figure 1.6 Overview of TCR recognition of peptide and metabolite presenting molecules. The top panels represent cartoon illustrations, indicative of the crystal structures of the ternary complexes: **a)** TCR-HLA-B and **(b)** MAIT TCR-5-OP-RU-MR1, respectively. The middle panels show in detail view of the antigen (Ag) binding site in MHC and MHC-like (MR1) molecules. The bottom panels indicate the molecular surface of MHC-I and MR1, alongside their bound Ags. Black-filled circles indicate the center of mass of the Va and Vb domains. CDR loops color codes: CDR1a, teal; CDR2a, pink; CDR3a, purple; CDR1b, red; CDR2b, orange; and CDR3b, yellow. Adapted from Awad, Wael; Le Nours, Jérôme; Kjer-Nielsen, Lars *et al* 2018.

1.4.2.3 - MR1 ligands and MAIT cell agonists and antagonists

The nature of the MR1 ligands was discovered and characterized by Kjer-Nielsen and colleagues, in which the authors proposed the first MR1 ligand capable of inducing MAIT cells activation[132,140,149]. Furthermore, it was postulated that MR1 can present riboflavin (Vitamin B_2) precursors, as well as folic acid synthesis pathways derivatives[140]. Curiously, depending on the level of MAIT cell activation in the presence of MR1 ligands, the latter can be differentiated in agonists and antagonists [148]. Starting with MAIT cell agonistic compounds, these derive from the riboflavin biosynthesis pathway, characteristic of plants, bacteria and fungi, and are denominated as ribityl lumazines[149,161,162]. Some examples of ribityl lumazines MR1 ligands are: reduced 6-hydroxymethyl-8-D-ribityllumazine (rRL-6-CH_2OH), 7-hydroxy-6-methyl-8-D-ribityllumazine (RL-6-Me-7-OH) and 6,7-dimethyl-8-D-ribityllmazine (RL-6,7-diMe)[149]. Further analysis on MR1 ligands was performed, as well as MAIT cell activation which led to the identification of different antigens (pyrimidines) that could work as MAIT cell agonists upregulating MAIT cell surface expression and activation rate. The most potent MAIT cell activatory ligands found to date are 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil (5-OE-RU) and 5-(2-oxopropylideneamino)-6-Dribitylamonouracil (5-OP-RU) [105,140,143]. These pyrimidic antigens are generated from a non-enzymatic condensation process between 5-amino-6-D-ribitylaminouracil (5-A-RU), identified by Corbett and colleagues as a key intermediate for MAIT cell activity and an early intermediate of the riboflavin synthesis pathway, with methylglyoxal and glyoxal byproducts[105,113,132,140,143]. Furthermore, since 5-A-RU contains a free amine that is susceptible to condensation reactions with metabolic byproducts and methylglyoxal/glyoxal are formed during metabolic pathways (for example glycolysis) in microbes and humans the end-products above mentioned are in constant production[105,113,143,163]. Despite being highly potent MAIT cell agonistic compounds, 5-OE-RU and 5-OP-RU are unstable and susceptible to condensation and consequent transformation in bicyclic ribityl lumazines[105,113]. However, when captured by MR1, these antigens are stabilized and function in a more potent manner in MAIT cell activation[105,113,149,157,164]. Besides MAIT cell agonistic MR1 ligands, there are also other MR1 ligands that despite upregulating MR1 cell surface expression lack the talent to activate MAIT cells. These compounds are described as MAIT cells antagonists, since they inhibit MAIT cell activation and are in constant competition with MAIT cells agonists for the MR1 binding pocket[113,132]. The first molecule identified as an MR1 ligand and MAIT cell antagonist was a non-stimulatory folic acid (vitamin B9)-related metabolite 6-formylpterin (6-FP)[143,149,155,165]. 6-FP is spontaneously produced due to the photodegradation of folic acid and is continuously bound to MR1.

Furthermore, instead of promoting MAIT cell activation similar to 5-OE-RU and 5-OP-RU, 6-FP inhibits or modulates MR1-mediated activation[113,114,133,166]. Following the identification of 6-FP, other pterin-based antigens were described such as acetyl-6-FP (Ac-6-FP) and two other variants of 6-FP (2-acetylamino-4-hydroxy-6-formylpteridine dimethyl acetal and 2-acetylamino-4-hydroxy-6-formylpteridine)[132,140,155,165]. These synthetic compounds were characterized as MAIT cell antagonists that inhibit MAIT cells activity, although are able to bind to MR1 and upregulate its cell surface expression levels[113,114,143,157,165]. Upon discovery, identification and characterization of MAIT cells

ligands, it became possible to generate MR1 tetramers with loaded with MAIT cells agonistic and antagonistic compounds, allowing specific detection and study of human MAIT cells in disease environments such as HBV like in this project[105,141,155,167,168]. Recently, it was identified a new panel of MR1 ligands which resembles a cocktail mix of drugs, drug metabolites and drug-like molecules. There are some examples of these new compounds like diclofenac (DCF) and salycylates that upregulate MR1 expression and at the same time promote the up or downregulation of MAIT cells[132,143,169]. Due to legal restrictions, commercial recombinant IL-12 and IL-18 were used to mimic the natural *in vivo* activation of MAIT cells in the liver, since these cytokines are produced by liver dendritic cells and monocytes during immune activation.

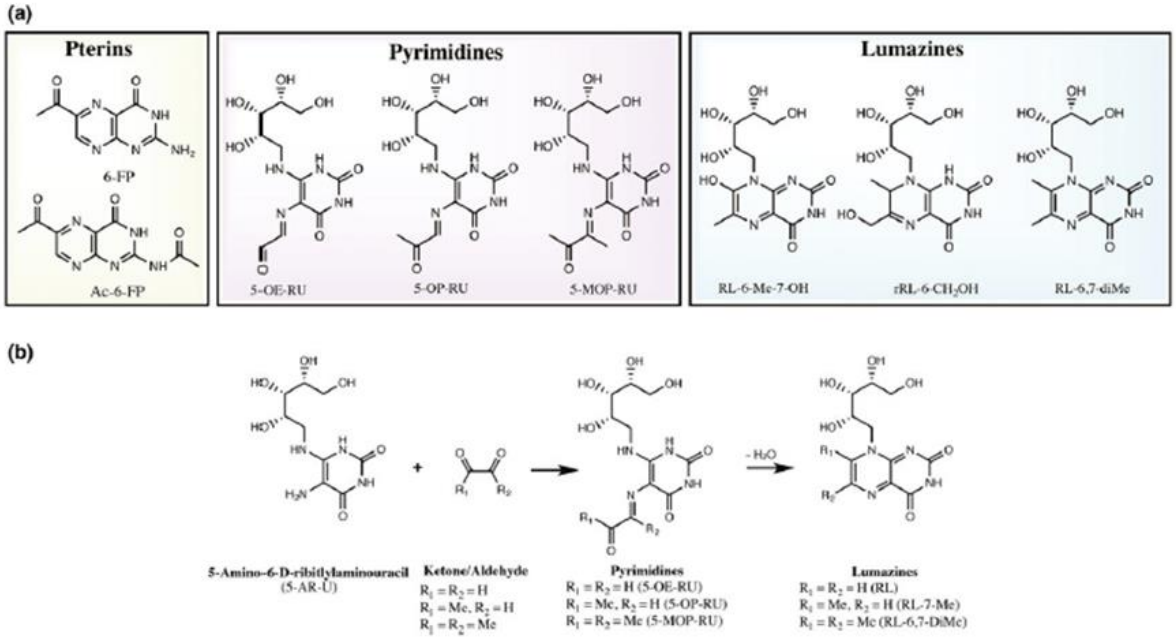

Figura 1.7 a) MR1-related Vitamin B2 and Vitamin B9 metabolite Ags chemical structures. Pterins: 6-formyl pterin (6-FP) and acetyl-6-formyl pterin (Ac-6-FP); Pyrimidines: 5-(2- oxoethylideneamino)-6-D-ribitylaminouracil (5-OE-RU), 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) and 5-(1-methyl-2-oxopropylideneamino)-6-D- ribitylaminouracil (5-MOP-RU); Lumazines: -hydroxy-6-methyl-8-D-ribityllumazine (RL-6-Me-7-OH), reduced 6-hydroxymethyl-8-D-ribityllumazine (rRL-6-CH2OH) and 6,7-dimethyl-8-D-ribityllumazine (RL-6,7-diMe). **b) Condensation reaction between** bacterially produced riboflavin biosynthesis intermediate 5-amino-6- D –ribityaminouracil (5-A-RU) with the reactive byproducts of metabolism glyoxal and methylglyoxal. There is a consequent production of the potent antigens 5-OE-RU and 5-OP-RU. These rapidly circularize to form ribityllumazines, which are far less potent than the pyrimidine antigens. . Adapted from Awad, Wael; Le Nours, Jérôme; Kjer-Nielsen, Lars *et al* 2018.

1.4.2.4 MAIT cell TCR recognition of MR1-ligand complexes

Previously, it was described that both MAIT agonists (5-OE-RU and 5-OP-RU), as well as antagonists such as 6-FP and Ac-6-FP binding mechanism to MR1 occurs[105,113,114,140,143,149]. The process itself takes place due to a covalent bond via the formation of a Schiff base between the ligand and the K43 residue within the MR1 structure[105,149,155]. Besides demonstrating a powerful link between MR1 and its ligands, also indicates that MR1 trafficking is directly influenced and triggered by the formation of a Schiff base and consequent molecular alterations, which allows MR1 cell surface expression increment[113,132,157]. In concordance with the statement above, several reports mention that the ribityl lumazine RL-6-Me-7-OH and DCF establish contact with the MR1 molecule but lack the strength to induce upregulation of MR1 cell surface expression, since no Schiff base is formed upon binding[105,113,143]. Furthermore, it is nowadays accepted that a few MAIT cell activating ribityl lumazines and pyrimidines resemble a MAIT cell antagonistic ligand (6-FP)[105,132,149,170]. However, within their structure, these ligands possess an extra ribityl moiety which promotes direct

contact between them and MAIT cells TCR[132,170,171]. Nonetheless, this is only possible due to the existence of a hydrogen bond between the ribityl moiety and a determinant residue (Y95) in the CD3α loop in MAIT cells TCR [105,170,172].

1.5 MAIT cells Effector Functions

1.5.1 – Cytokine Production

MAIT cells upon activation, besides producing pro-inflammatory cytokines[173] they also respond to microbial stimulation, riboflavin-producing microbes, which response is translated in the upregulation of activation markers such as CD69 and CD25 (IL-2Rα chain)[106,132,160,174–176]. On the other hand, microbes that lack the riboflavin synthesis pathway[149,173], like *Escherichia faecalis*, can still activate MAIT cells due to the presence of IL-12 and IL-18 markers as described below in MR1-dependent/independent MAIT cell responses[107,130,132]. As it happens during T cells stimulation and activation, also in MAIT cells there is a presence of Th_1[115,173,177,178] and Th_2 cytokines families[115,132,179,180] , which may influence the activation rate of these cells, as well as increase or decrease specific cytokines expression. These cells are well-described for their function within the immune system, where they seem be responsible for cell-mediated immunity and strong antibody production[181]. The goal of these T helper cells is to provide phagocyte-dependent and independent protective responses against different kinds of pathogens[106].

It has previously been reported that peripheral blood MAIT cells produce high levels of IFN-γ and TNF-α, these two pro-inflammatory cytokines being part of the Th_1 cytokine family[123,132,141,159,160,173,178]. On the other hand, a subset this cytokine family, Th_{17} cytokines, appear to be expressed in a lower or inexistent degree[115,131,174]. Cytokines such as IL-17A and IL-22 are included in this cytokine family and its expression may depend on the MAIT cell population tissue localization analyzed[173,177]. Despite this, upon stimulation with strong stimuli such as PMA and ionomycin[115,131,132,173], peripheral blood MAIT cells these cells start secreting IL-17A, even though at lower levels than liver MAIT cells[131,132,152]. Also, in comparison to Th1 and Th17 cytokines, the production of Th2 cytokine family, which includes IL-4, IL-5, IL-9 and IL-13 cytokines, as well as IL-10, a known T cell regulatory (Treg) cytokine are likely expressed by peripheral blood and liver MAIT cells in a minimal degree[131,132,179,180].

As mentioned previously, when MAIT cells are stimulated with PMA/Ionomycin, specific cytokines show an upregulated expression, compared to unstimulated samples. Thus, superantigens[182] , can also be used as potent stimuli to induce MAIT cells activation and cytokine production by these cells. The latter are exotoxins secreted by bacterial species that cross-link TCRs in T cells, as well as MHC class II molecules on APCs[132,182]. Therefore, MAIT cell activation pattern becomes enhanced and the release of pro-inflammatory mediators increases[132,183,184]. As an example, it is described that MAIT cells in adipose tissues can indeed secrete high levels of IL-10[173] or that it is possible to detect IL-2 expression levels in liver and peripheral blood MAIT cells but only after PMA/Ionomycin[115,131,132,141] or superantigens stimulation[132,182]. It appears to exist an involvement by MAIT cells in cross-talk mechanisms with other cells types, like APCs, and this might be an explanation to why activated MAIT cells produce granulocyte-macrophage colony stimulating factor (GM-CSF) and why MAIT cells can work as mediators of survival, activation and differentiation of other cellular types or T cells subsets [132,185,186].

1.5.1.1 – Degranulation and Cytotoxicity

As one of the hallmarks of MAIT cells is to recognize infected target cells and kill them, it is reasonable that being a subset of T cells, these cells have the same effector functions[106,132,173]. However, MAIT cells might have evolved in such a manner that they could improve this ability by secreting different cytokines, as well as regulating their cytotoxicity role. This cellular mechanism takes place for the most part via granule exocytosis and it is built on the effector cells elements that facilitate the elimination of the target cells[173].

Subsequently to microbial stimulation, besides producing a various number of cytokines, MAIT cells are also described as key players in degranulation and being able to kill infected target cells[132]. While evaluating MAIT cells degranulation capability and killing rate it was discovered that peripheral blood MAIT cells present specific cytokines that lead to the death of infected target cells[106,140,176,187]. Therefore, holding a significant and possible new cell based therapeutic approach based on its effector functionality, despite effector MAIT cells expressing programmed cell death protein 1 (PD-1), a known exhaustion marker[188]. Peripheral blood MAIT cells, *ex vivo*, are able to express (once activated) granzyme A (Grz A), residual levels of granzyme B (Grz B), low levels of perforin (Prf) and granulysin (Gnly), which levels are variable, probably due to inner-donor variability or intrinsic fluctuation in this specific cell type[115,132,140,173,176,187]. The above cytokines mentioned might influence MAIT cells ability and function upon the presence of infection and allow these cells to directly encounter infected cells and to kill them. Prf is a pore-forming protein that consents the discharge of granzymes and Gnly molecules to the cytoplasm[132,189]. The latter cytotoxic effector molecules are recognized for their direct link to cytotoxic $CD8^+$ T cells[173]. Therefore, imposing enough influence on MAIT cells and enabling these cells to induce apoptotic mechanisms on infected target cells[132,140,189]. Lastly, Gnly is an antibacterial protein recognized for killing intracellular bacteria by disrupting their membranes[132,189,190]. This indicates that MAIT cells contain a readily available pool of cytotoxic molecules (Prf/Grz A/Gnly) that can be rapidly released upon degranulation[132]. After activation, MAIT cells are subjected to a degranulation mechanism where CD107 A, a type-I transmembrane protein familiar for its function in degranulation in lymphocytes, is upregulated. In contrast, while activated, MAIT cells undergo a Grz A and Gnly expression downregulation. Nevertheless, when stimulated in a MR1-dependent manner *in vitro*, MAIT cells are able to lyse bacteria-infected epithelial cells[176] and *E. coli*-infected THP1 cells [173,187,191], as well as *S. flexinirii* infected HeLa cells expressing endogenous levels of MR1[106,176]. This suggests that MAIT cells improve their degranulation and cytotoxicity effector functions, upon stimulation. Also, further studies are needed to compare if MAIT cells transduced with HBV TCR produce as much Prf/GrzA/Gnly as MR1-dependent activation, for greater clarity in the efficacy of TCR transduced MAIT cells.

1.5.1.2 – Proliferation

The Ki-67 marker/protein is a well-described marker strongly associated with cellular proliferation[192]. Proliferation of pathogen specific immune cells are a vital step in controlling infection, and is one of the first functions lost during T cell exhaustion. Furthermore, clonal proliferation is a force-multiplier for the immune response, especially in rapid pathogen growth Despite the apparently inexistence of basal levels of Ki-67 expression *ex-vivo*, peripheral blood MAIT cells are still able to proliferate *in vitro* upon microbial response[115,177,187]. This response might be strongly related to their response to riboflavin, vitamin B_2, and other bacterial products[149]. As mentioned previously, upon *in vitro* stimulation MAIT cells increase their proliferation ability due to an upregulated expression of the Ki-67 marker[177]. Nonetheless, MAIT cells seem to keep their

cytolytic profile, whereas displaying high GrzB and Prf expression levels, known for their role in degranulation and cytotoxicity in MAIT cells effector functionalities[106,132,187].

1.5.1.3 – MR1 – dependent/independent MAIT cell responses

One parameter that influenced the insight about MAIT cells was the discovery of its ligands, provided as a possible explanation to MAIT cells unresponsiveness when stimulated by bacteria that lack riboflavin metabolites, but becoming actively functional when presented to MR1 molecules inducing MAIT cells activation[149]. MR1 is a non-polymorphic gene located on chromosome 1 in humans[142], similar to the CD1 gene[193], and outside of the MHC located on chromosome 6 [132,194]. As MR1 is expressed ubiquitously and different cell types, such as DCs [109,195], B cells[178] and macrophages[123] can behave as APCs, it became easier to evaluate MAIT cells effector functionalities, since these cells function in a similar manner[132,154]. Also, scientific studies on these cells led to the conclusion that MAIT cells activation upon microbial stimulation is directly correlated to TCR antigens and MR1 interactions, which are considered MR1-dependent MAIT cell responses[148,196] (**Figure 1.6**). Cytokine production (IFNγ and TNFα), degranulation, cytotoxicity, proliferation are examples of effector functions associated to MR1-dependent TCR interactions[123,131,176,178,187,197]. Besides these, upregulation of GrzB and Prf, as well as production of IL-22[118,187], and the IL-10 cytokine, a family member and known marker for cellular inflammatory response, are responsible for MAIT cell activation in a MR1-dependent manner. Another criteria used to define MR1-dependent TCR MAIT cell responses is based on these cells response to microbial response, in particular to bacteria which display the riboflavin biosynthesis pathway such as *Escherichia coli*[107]. Nonetheless, there are bacterial species, for example *Escherichia faecalis*, that lack this pathway and are not able to induce an MR1-dependent activation response on MAIT cells[106,107,130,132]. In resemblance to other innate-like cells such as NK T cells, MAIT cells can also be directly activated by lymphokines in response to an infection in an MR1-independent manner[106,107,173,198]. As an example, *in vitro* studies using cells infected with *Mycobacterium bovis* Bacillus Calmette–Guérin (BCG) have shown to induce solely MR1-independent MAIT cell activation responses[173]. Also, MAIT cells are known for expressing IL-12R and IL-18R receptors[123,124,160], and when stimulated with innate-like cytokines, IL-12 and IL-18, they induce MAIT cells activation responses in a MR1-independent manner and without TCR signaling influence[107,132]. Supporting this fact, Ussher and colleagues[107] performed experiments regarding MAIT cells cytokine-mediated activation and reported that MAIT cells could produce IFNγ after TCR blocking and in culture with IL-12 and IL-18[173]. Besides IL-12 and IL-18 there is evidence of other cytokines such as IL-15 and IL-7 being able to activate MAIT cells in a MR1-independent process[106,173,199]. IL-15 appears to partake in a synergistic relation with IL-12 and/or IL-18, which translates the activation of peripheral blood MAIT cells even without microbial stimulation[132,199]. In addition, IL-15 directly potentiates IFNγ secretion, cytolytic activities (GrzB production) and induces MAIT cells expression of the early activation marker CD69 through the same mechanisms after sub-optimal TCR triggering[106,182,200,201]. Furthermore, IL-7 is implied in MAIT cell effector functions and is yet considered an extra marker to bear in mind in MR1-independent MAIT cell responses to innate cytokines[148,196]. Le Bourhis L and colleagues[176] reported that MAIT cells from the liver highly express IL-7R, hence IL-7 potentiates TCR-dependent secretion of Th_1 cytokine and of IL-17A[173,176]. This innate-like cytokine is also directly correlated with an augmented expression of GrzB, Prf and several transcription factors (PLZF, RORγt, T-bet, Eomes and Helios), which are of extreme importance effector molecules in MAIT cells stimulatory activation mechanisms[202]. Curiously, IFNγ, TNFα and IL-17A, important during bacterial infections, expression after IL-7 stimulation seems to be nonexistent[152,176]. MAIT cells are indeed a very remarkable T cell subset that undergoes cytokine mediated activation and not only can respond to bacterial infection, while this

capacity seems to allow them to respond to certain viral infections. Regarding viral infections, MAIT cells are suggested to be indirectly activated due to a cytokine-mediated stimulation mainly caused by the secretion of IL-12 and IL-18 by parenchyma or hematopoietic cells[106,182,203]. Supporting this statement, several reports with IL-12, IL-15 and IL-18 blocking experimental procedures showed that IFNγ production by MAIT cells in viral infections such as dengue virus, influenza virus and Hepatitis C virus (HCV) is directly affected and depends on IL-12 and IL-18, IL-18 alone, and IL-18 and IL-15, respectively [132,182,203]. *In vitro* studies using TLR agonists suggest that MAIT cells are activated independently of microbe or virus stimulation, as well as in a MR1-independent manner[107,130]. For example, agonists for TLR3, TLR4 (lipopolysaccharide, LPS) and TLR8 (single-stranded RNA40) indirectly through APC's activate peripheral blood MAIT cells and induce IFNγ production via IL-12 and IL-18 stimulation [107,109,130,132].

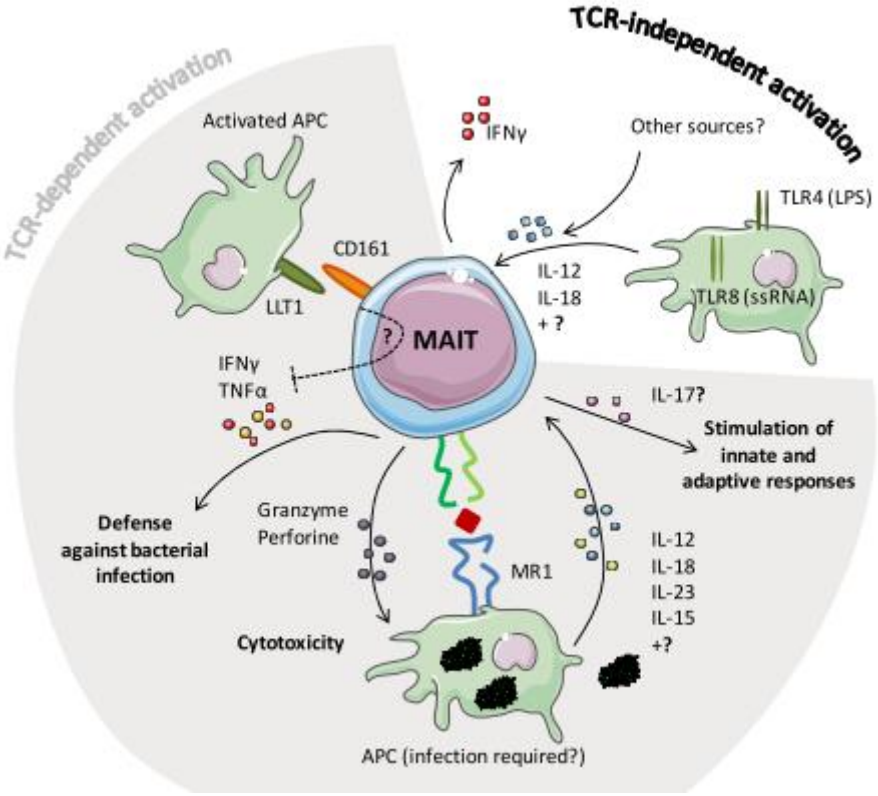

Figure 1.8 MAIT cells activation in a TCR-dependent or –independent manner. TCR engagement triggers secretion of mainly IFNγ and TNFα by MAIT cells. Although MAIT cells express CD161, IL-17 production can also be achieved upon PMA/ionomycin stimulation. MAIT cells can also secrete cytotoxic effectors and kill infected cells. These cells secretion profile is strongly supported by their role in immune defense against pathogens. MAIT cells effector functions can be modulated through different pathways. CD161 triggering can be acknowledged as one of the modulators of MAIT cells effector functions, since its expression regulates consequently decreases the amount of IFNγ and TNFα produced, but not the level of cytotoxic molecules secreted. Among other cytokines, IL-12, IL-18, IL-23, and IL-15 that are produced for example by infected cells, can modulate the cytokine secretion profile. As for MAIT cells activation through TCR-independent mechanisms, IL-12 and IL-18, secreted by liver and blood monocytes appear to have a pivotal participation in TCR-independent MAIT cells activation. Adapted from Franciszkiewicz et al 2016

1.5.1.4 - Regulatory Aspect of MAIT cells effector functions

As described in this chapter, it is strongly suggested that MAIT cells are involved in a give-and-take relationship with other cell types, as seen in studies performed in MAIT cell activation using APCs[204]. Also, microbes and innate cytokines stimulations seem to regulate MAIT cells activation responses through different mechanisms and mediators[205]. In one single study, it was reported that MAIT cells can be actively induced merely by the presence of innate cytokines such as IL-12 and IL-18 and when combined with TCR stimulation, MAIT cells show an increased stimulatory response[201]. However, it seems that MAIT cells are not functionally activated upon TCR stimulation alone[201]. MAIT cells are also characterized for expressing numerous membrane molecules such as CD161, NKP30 and NKG2D[106]. These membrane molecules function as immune-modulators and prevent inflammatory responses to commensal riboflavin biosynthesis-competent microorganisms [115,132,141]. Interestingly, only CD161 triggering mechanism has been studied[176,206] and depending on which cellular type this lectin-like membrane receptor is expressed it was concluded that CD161 can be inhibitory or exert an activator function[106,207]. This was proved in studies using NK and $CD8^+$ T cells [208–210]. Currently, it is suggested that MAIT cells responses can be modulated on CD161 expression. However, this modulation is quite controversial, since different studies report different results. On one side, CD161 presence seems to downregulate the expression of activation markers and cytokine production, without affecting MAIT cells cytolytic effector functions[201], whereas other studies report an increase in cytokine production[176].

In any case, most of the science performed regarding MAIT cells effector functions uses human peripheral blood mononuclear cells (PBMCs), instead of liver and mucosal tissues samples, where these cells can be found at a higher percentage[106]. Therefore, it would be of the utmost interest to increase the number of studies on MAIT cells, sourced from more disease relevant organs as mentioned previously.

Chapter 2 – Objectives

The main goal of this project is to phenotypically characterize MAIT cells. Here, our focus is to compare MAIT cells isolated from PBMCs from both healthy and CHB patients and to perform a comparative analysis that could be indicative of a potential role of MAIT cells as a new immunotherapeutic approach for infectious diseases such as HBV. The specific goals of the project are the following:

- **To address whether there are differences in the phenotype of MAIT cells in the peripheral blood from healthy *versus* CHB patients.** For this, we will evaluate the percentage of total MAIT cells, as well as the percentage of $CD4^+$, $CD8^+$ and $CD56^+$ MAIT cells through flow cytometry analysis.

- **To investigate whether there are functional differences within MAIT cells isolated from healthy *versus* CHB patients.** For this purpose, we will analyze the percentage of MAIT cells that are positive for 1) checkpoint inhibitors PD-1, TIM3 and LAG3, 2) activation markers CD25, CD38, CD69 and HLA-DR and 3) pro-inflammatory cytokines IFNγ, TNFα and IL-2. As in the previous objective, we will employ flow cytometry for this comparative analysis. Additionally, we will evaluate the cytokine and chemokine expression profile of these MAIT cells when in presence of APCs. To achieve this, isolated MAIT cells will be combined with a B lymphoblast cell line (HMy2.CIR) which mimics the activity of APCs, and a $CD8^+$ cytotoxic T cell Luminex Kit will be performed using these cells.

In parallel, we will also perform a **serum screen to evaluate the performance of commercially available serums in immunological assays**. In the scope of this objective, we will use ELISpot, ICS and proliferation assays in PBMCs form different healthy patients, in order to find the serum that provides the lowest background levels and highest signal amplification in each of these assays. Results will be directly compared to a currently in use *in-house* serum, and the chosen serum will be used in further experiments performed in our lab.

Chapter 3 – Materials and Methods

3.1 Human Samples

3.1.1 Healthy Donors

Buffy coats of healthy patients were provided by Belgium Red Cross with the proper patients consent and according the Belgium law for the use of human primary materials.

3.1.2 CHB Patients

All patient samples were collected by Caprion Biosciences (Montreal, Canada), with the written informed consent of all patients and according to all legal regulations for the handling of human primary materials. In **Table 3.1**, an overview of the patient chronic status can be found. They were all chronic HBV patients based on high levels of HBsAg and no detectable levels of HBs antibodies. All patients are currently on treatment.

Table 3.1. Overview Patient Disease status

Chronic HBV Donors								
Subject ID	**Chronic Hepatitis B**	**HBsAg QT**	**Anti-HBs**	**HBeAg**	**Anti-HBe**	**Anti-HBc**	**HBV DNA**	**ALT**
HBV 1	Y	5564.57	0	Reactive	Non-Reactive	Reactive	< 20	19
HBV 2	Y	5019.49	0.13	Non-Reactive	Reactive	Reactive	< 20	44
HBV 3	Y	7327.7	Non-Reactive	Reactive	Non-Reactive	Reactive	303	28

3.1.3 Isolation of Peripheral Blood Mononuclear Cells

Peripheral Blood Mononuclear Cells (PBMCs) were isolated from buffy coats through a density gradient centrifugation by using Lymphoprep™ (Axis-Shield, Cat. 1114547) (**Figure 3.1**). After 3 washes with medium, cells were counted on GUAVA (using GUAVA ViaCount™ Flex Reagent) or on MoxiFlo ORFLO (using Moxi Cycle Viability Reagent, Cat. MXA055). Cells were cryopreserved in the presence of 10% Dimethylsulphoxide (DMSO) (Sigma - Life Science, Cat. D2650) and 90% heat-inactivated fetal calf serum (FCS) (GIBCO, Cat. 1865043), and aliquoted in cryovials. Subsequently, the vials were placed in freezing boxes, filled with 100% isopropanol (Mister Frosty), and placed at -80^{0}C (at least overnight) to slowly freeze the cells. After that, vials were transferred to the liquid nitrogen (-175ºC) tanks for long-term storage until usage in subsequent assays.

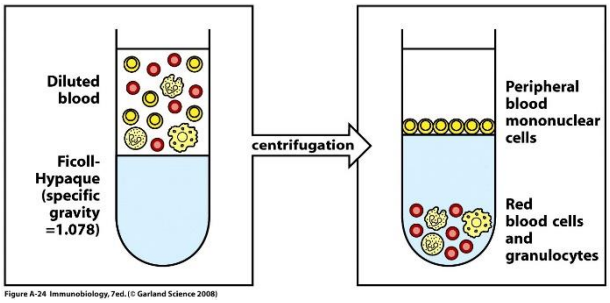

Figure 3.1.. Overview of steps in isolation of Peripheral Blood Mononuclear Cells (PMBCs). *Murphy, K., Travers, P., Walport, M., & Janeway, C. (2012).* Janeway's immunobiology *(8th ed.). New York: Garland Science.*

3.1.4 Thawing of the PBMCs

The cryovials were thawed by transferring them directly from liquid nitrogen to a water bath (37ºC). The cells were washed 3 times with RPMI-1640 medium (Lonza, Cat. 12-702F) supplemented with 10% fetal calf serum (FCS) (GIBCO, Cat. 1865043) and 1% L-Glutamine (Sigma - Life Science, 200mM, Cat. G8541) to remove all the cryoprotectant DMSO and allowing cell recovery from the freezing process to prevent reduction in viability in following biological assays.

3.2 Mucosal-Associated Invariant T cells (MAIT cells)

3.2.1 Flow Cytometry

Flow cytometry is a well-established laser-based technique used to count, sort and phenotype different cell subpopulations within an heterogenous cell population (**Figure 3.2**). Using a panel of specific antibodies, each conjugated with a distinct fluorescent dye, fluorochrome, different cell subpopulations can be distinguished based on a combination of colors emitted by the cells upon excitation with multiple lasers in the flow cytometer. Cell populations are also distinguished based on their size (Forward-Scatter), granularity (Side-Scatter). The labeled samples go through a liquid stream where they are passing through a laser beam. This excites the fluorochromes which will emit light at a certain wavelength.

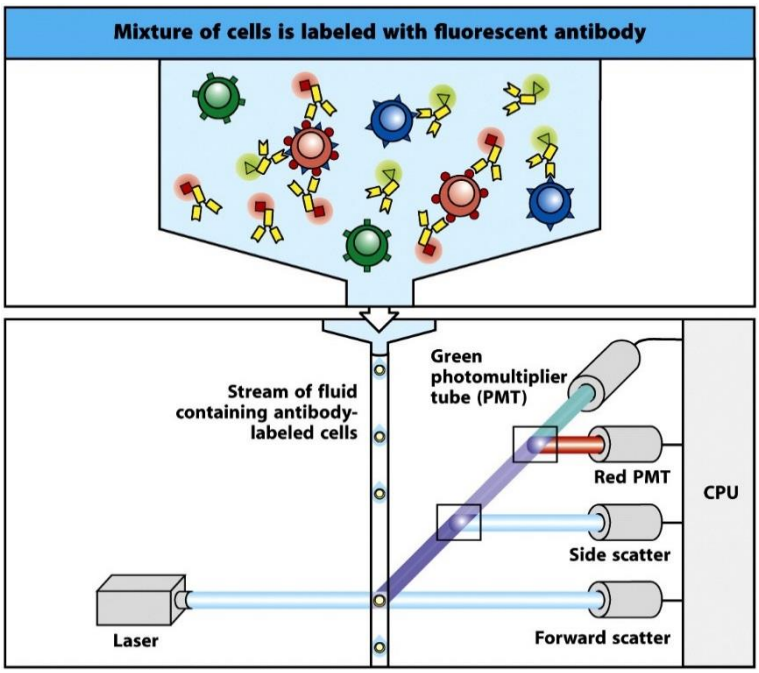

Figure 3.2. Flow Cytometry Technique Principle Overview - *Murphy, K., Travers, P., Walport, M., & Janeway, C. (2012).* Janeway's immunobiology *(8th ed.). New York: Garland Science.*

This technique enables us to quantify and evaluate specific cell populations functionalities based on activation markers, cytokine secretion and proliferation parameters, among others. In the context of this project, flow cytometry was used to identify, characterize and compare mucosal-associated invariant T (MAIT) cells between healthy and chronically infected hepatitis B patients. For this purpose, cellular surface and intracellular markers were used that would allow us to explore the phenotypic profile of MAIT cells between healthy and chronic HBV patients. Besides the lineage markers to define the MAIT cells, this evaluation was done based on 3 antibody panels: 1) pro-inflammatory cytokine production, 2) activation markers and 3) checkpoint inhibitors (**Table 3.2**).

Processed PBMCs were stained with LIVE/DEAD staining carried out using Fixable Viability Dye 660 (eFluor™) (ThermoFisher, Cat. 65-0864-14) dissolved in PBS (Dulbecco's Phosphate Buffered Saline, Sigma-Life Science, Cat. D8537) and applied to all samples as a viability marker. This LIVE/DEAD stain mechanism is based on the reaction of a fluorescent reactive dye with cellular proteins (amines). The dye is incapable of penetrating into live cells membranes. However, this reactive dye can permeate membranes from dead cells irreversibly, staining amines, located both on the interior and exterior of the membrane.

Surface antibodies were diluted in Horizon Brilliant™ Stain Buffer (BD Bioscience, Cat. 563794) per BD instruction for Horizon Brilliant stain buffer (Panel 1-3). This buffer prevents conjugation of the BV dyes. Afterwards samples were incubated in the dark at 4ºC for 30 minutes. After washing the samples and prior to the addition of intracellular antibodies (Panel 1), cells were

fixed with CytofixTM (4.21% formaldehyde w/w and saponin) (BD Bioscience, Cat. 555028) in the dark at 4ºC for 20 minutes to crosslink, stabilize and fix the cell membrane. Brefeldin A (GolgiPlug™ BD Bioscience, Cat. 555029) was added to block intracellular protein transport processes so that cytokines can accumulate in the Golgi complex and are prevented to be secreted.

Further on, these samples were washed with Perm/Wash™ buffer (BD Bioscience, Cat. 554723) containing saponin (diluted 10x in distilled H2O) to permeabilize the cell membranes. Intracellular staining antibodies for panel 1 were diluted in Perm/WashTM buffer and added to the cells following an incubation in the dark at 4ºC for 30 minutes.

Table 3.2. Antibodies used during flow cytometry experiments

Antibodies Common To Each Panel							
Marker	**Fluorochrome**	**Clone**	**Vendor**	**Catalog**	**AB**	**Volume per Sample (µL)**	**Staining**
CD3	AF 488	UTCH1	BD	557694	M	5	Surface
CD56	BB700	NCAM 16.2	BD	745894	M	5	Surface
CD161	BV421	DX12	BD	562615	M	5	Surface
TCRVα7.2	APC-Vio770	REA179	Milteny	130-100-179	?	10	Surface
Panel 1 - Pro-Inflammatory Cytokines							
Marker	**Fluorochrome**	**Clone**	**Vendor**	**Catalog**	**AB**	**Volume per Sample (µL)**	**Staining**
CD4	Bv650	RPA-T8	BD	563795	M	5	Surface
CD8	BUV395	RPA-T4	BD	740563	M	5	Surface
IFN-g	PECF594	B27	BD	562392	M	5	Intracellular
TNF-α	PE-Cy7	MAb11	BD	560678	M	5	Intracellular
IL-2	BUV737	MQ1-17H12	BD	564446	R	5	Intracellular
Panel 2 - Activation Markers							
Marker	**Fluorochrome**	**Clone**	**Vendor**	**Catalog**	**AB**	**Volume per Sample (µL)**	**Staining**
CD25	BUV395	M-A251	BD	740290	M	5	Surface
CD38	BV711	HIT2	BD	563965	M	5	Surface
CD69	PE-Cy7	L78	BD	335792	M	5	Surface
HLA-DR	PECF594	G46-6	BD	562304	M	5	Surface
Panel 3 - Checkpoint Inhibitors							
Marker	**Fluorochrome**	**Clone**	**Vendor**	**Catalog**	**AB**	**Volume per Sample (µL)**	**Staining**
PD-1 (CD279)	BUV737	EH12.1	BD	565299	M	5	Surface
TIM-3 (CD366)	BV711	7D3	BD	565566	M	5	Surface
LAG-3 (CD223)	BUV395	T47-530	BD	745640	M	5	Surface
γDTCR	PECF594	B1	BD	562511	M	5	Surface

ArC™ Amine Reactive Compensation Beads (ThermoFisher Scientific, Cat. A10346, Lot 1703807) were incubated and used as a compensation control for live/dead stain. BD™ CompBeads Positive Control (BD Bioscience, Cat. 51-90-9001229) and BD™ Negative Control (BD Bioscience, Cat. 51-90-9001291) Compbeads incubated with a single antibody were used as compensation controls for all fluorochromes, except for the IL-2 and TCRVα antibodies. BD™ CompBeads Anti-Rat/Hamster Ig, κ (BD Bioscience, Cat. 51-90-9001189) and UltraComp eBeads (eBioscience™, Cat. 01-2222-42), were used for IL-2 and TCRVα antibodies, respectively. Every experimental procedure regarding flow cytometry, MAIT cells marker's expression were performed by plating 1 million PBMCs in each well, already supplemented with FCS serum. Afterwards cells were incubated and stimulated for 8 hours at 37ºC.

All data acquisition was executed on a BD Biosciences LSR Fortessa™ flow cytometer with a 5-laser configuration (355-, 405-, 488-, 561-, and 639-nm lasers). The data generated was processed with BD FacsDiva v.8.0.1, Cytobank Janssen, FlowJo v. 10 and TIBCO Spotfire software. Fluorescence Minus One (FMO) control staining was integrated within the experiments to establish the gating strategy.

3.3 Gating Strategy of Mucosal-Associated Invariant T (MAIT) cells:

A gating strategy was developed to identify MAIT cells within the PBMC population and characterize them phenotypically, while using MAIT cells specific marker, CD161 and TCRVα7.2. Initially, the PBMCs samples were gated for lymphocytes, single cells and living cells. Afterwards, and within the living cell population, the samples were gated on the $CD3^+$ T cells. Within this population, MAIT cells were gated and identified by using two different markers: CD161 and TCRVα7.2 (**Figure 3.3**). All other markers (**Table 3.2**) were gated after, as phenotypic markers for MAIT cells.

PBMCs were stimulated with IL-12p70 (Human IL-12p70 Recombinant Protein, ThermoFisher, Cat. 14-8129-62) and IL-18 (Recombinant Human IL-18-1F4 Protein, R&D Systems, Cat. 9124-IL-010), used as activation stimuli for MAIT cells. Polyclonal stimulus phytohemagglutinin (PHA) stimulation (Sigma, Cat. L4144) stimulation and anti-CD3/CD28 BD Beads (Gibco, ThermoFisher Dynabeads™ Human T-activator CD3/CD28, Cat. 11453D) were used as positive

Figure 3.3. Gating strategy used to identify and characterize MAIT cells within PBMC's samples

controls, while DMSO stimulus (Sigma - Life Science, Cat. D2650) and unstimulated samples were used as negative control.

3.4 Isolation of MAIT cells

3.4.1 Isolation of MAIT cells with Microbeads Separation Kit

PBMCs were thawed and counted as previously mentioned (See **3.1.4 Thawing of the PBMCs**). The PBMC culture was rested overnight at 37ºC, in a 5% CO_2 incubator to let the cells recover from the thawing process. On the following day, the cells were isolated using two different cell markers (CD161 and TCRVα7.2), as well as two distinct multisort microbeads separation kits: anti-PE Multisort Kit (MiltenyiBiotec, Cat. 130-090-757) and anti-APC Multisort Kit (MiltenyiBiotec, Cat. 130-091-255). These kits are commercially available and are used for sorting different cell populations according to specific surface markers due to an indirect magnetic labeling system. Then, these cell suspensions go through a MACS-MS column to be sorted. Alongside with the separation kits mentioned above, additional experiments included a Dead Cell Removal Kit (MiltenyiBiotec, Cat. 130-090-101) as a complementary kit, which allows the removal of dead cells during beads cell isolation. The separation was performed in FACS tubes and the cell suspension was stained with two different antibodies, anti-CD161 (PE, BD Bioscience, Cat. 556081) and anti-TCRVα7.2 (APC, MiltenyiBiotec, Cat. 130-100-177). Along the isolation procedure, samples were taken at different timepoints, transferred to a 96-well plate and stained with LIVE/DEAD Fixable Viability Dye 780 (eFluor™) (ThermoFisher, Cat. 65-0865-14) and anti-CD3 (Alexa Fluor 488, BD Bioscience, Cat. 557694). Anti-CD56 (BB700, BD Bioscience, Cat. 745894) was used as a control measurement. All data acquisition was performed on a BD Bioscience LSR Fortessa™ flow cytometer.

3.4.2 FACS Cell-Sorting

PBMCs were thawed and counted as previously described (See **3.1.4 Thawing of the PBMCs**) as an initial viability check to confirm the quality of PBMC samples (>70% viability). Following this, the PBMC cell suspension was centrifuged, diluted in PBS (Dulbecco's Phosphate Buffered Saline, Sigma-Life Science, Cat. D8537), stained LIVE/DEAD viability using Fixable Viability Dye 660 (eFluor™) (ThermoFisher, Cat. 65-0864-14) and incubated in the dark at 4ºC for

30 minutes. Afterwards, cells were stained for anti-CD3, anti-CD161 and anti-TCRVα7.2 (**Table 3.2**) and incubated in the dark at 4ºC for 30 minutes. MAIT cells were sorted from the PBMC population using a BD Bioscience FACS Aria1™ cell sorter. Differing from the preciously used FACS Fortessa™ this cell sorter is equipped with 3 lasers (405-, 488- and 633-nm) wavelengths. Importantly, since it is a time-consuming process (cells might die), sorted MAIT cells were directly transferred to RPMI-1640 medium (Lonza, Cat. 12-702F) supplemented with 50% % fetal calf serum (FCS) (GIBCO, Cat. 1865043) and 1% L-Glutamine (Sigma - Life Science, 200mM, Cat. G8541) via an automatic process to improve the cells' viability. The sorted cell suspension was put at 37ºC in a 5% CO_2 incubator for overnight resting.

3.5 Functional Assay – C1R Cell line

3.5.1 Cell Culture

The B lymphoblast cell line, HMy2.CIR [C1R, HMy2.C1R] (ATCC® CRL1993™), was cultured in IMDM (1x) – Iscove's Modified Dulbecco's Medium (GIbco, Cat. 12440-053) supplemented with L-glutamine and 25mM HEPES and 10% FCS as mentioned by ATCC instructions. Cells were split every 2 to3 days and seeded with a concentration between $2*10^5$ and $1*10^6$ cells per mL of medium, according to the manufacture instructions. After passage 3, a mycoplasma test sample was taken from the cell line suspension to check for possible mycoplasma contaminations.

3.5.2 Stimulation of Sorted MAIT cells in combination with B Lymphoblast cell line

The B lymphoblast cell line, HMy2.CIR [C1R, HMy2.C1R] (ATCC® CRL1993™), performing as antigen presenting cells (APCs), and MAIT cells, previously sorted (See **3.4 Isolation of MAIT cells**), were stimulated in 3 different ratios, 2:1, 5:1 and 10:1 d for 20 hours at 37ºC with 5% CO_2 with different stimuli. These included PHA (Sigma, Cat. L4144), lipopolysaccharide (LPS, InvivoGen, Cat. tlrl-eklps), two concentration levels (0.5 and 0.25%) DMSO (Sigma - Life Science, Cat. D2650) and two different concentrations (25μM and 50μM) of Diclofenac, an MR1 agonist. The next day supernatant was collected and frozen at -80ºC for Luminex assay read out. Cells were also checked for activation markers using flow cytometry technology.

3.5.3 Flow cytometry

Samples were stained for LIVE/DEAD viability marker, common markers and activation markers (See **3.2.1 Flow Cytometry and Table 3.2**). Due to the presence of an B lymphoblast cell line an extra antibody was used, CD20 (FITC, BD Bioscience, Cat. 345792), to exclude B cells from the flow cytometry analysis. As CD20 antibody used FITC fluorescence channel necessary for detection, CD3 fluorescence channel was switched to PerCP-Cy5.5 (BD Bioscience, Cat. 557694). All data acquisition was performed on a BD Bioscience LSR Fortessa™ flow cytometer.

3.5.4 Luminex

Luminex is a multi-analyte profiling based technology that enables the detection and quantification of numerous secreted proteins (cytokines and chemokines) by testing one single sample. This immunoassay instrumentation displays high efficiency levels, and dynamic range for the number of analytes that can be tested in one sample. Luminex sample reader is composed by two lasers (Green – 532nm and Red – 635-nm), that when combined with different labeled beads (magnetic/polystyrene) can generate a combination of 80 individual color combinations. The Luminex sample reader also contains a fluidic and real-time digital quantification signal processing. These color-coded beads are pre-coated with analyte-specific capture antibodies, one per bead, for the molecules of interest. This leads to the capture of analytes of interest by these analyte-specific capture

antibodies. Phycoerythrin (PE)-conjugated streptavidin is also added and will bind to the biotinylated detection antibodies. Following the addition of specific biotinylated detection antibodies, they will bind to the analytes of interest forming what is commonly called as antibody-antigen sandwich.

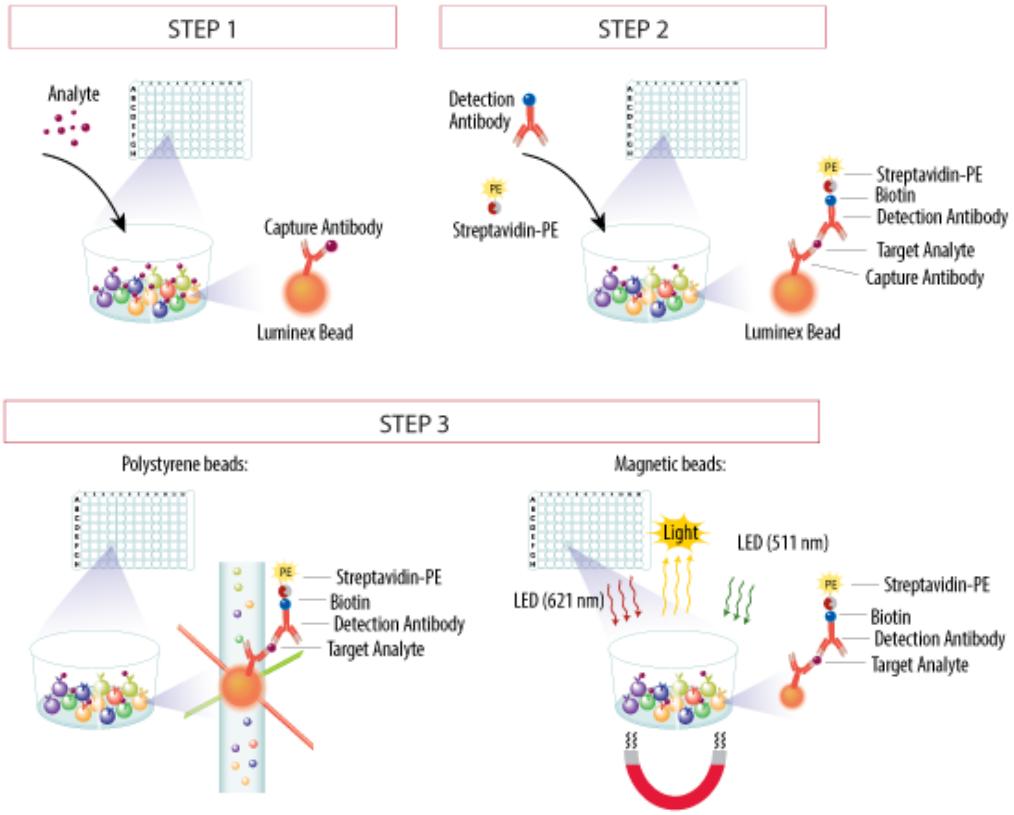

Figure 3.4. Luminex Principle Overview – From https://www.rndsystems.com/resources/technical/luminex-assay-principle

When the analytes are crossed by the laser beams, they are analyzed and quantified based on the conjugation between the specific antibodies – beads complex formed. However, this technology has the disadvantage of not being able to discriminate the cell type or cell population that produced a specific cytokine/chemokine (**Figure 3.4**).

For this project, this assay was employed to quantify and evaluate the type of cytokines and chemokines expressed by sorted MAIT cells upon stimulation (**Table 3.3**). For that purpose, we used a CD8 cytotoxic T cell Luminex Kit (Milipore, previously qualified by Janssen Lab). Undiluted supernatant from the functional assay performed with the B lymphoblast cell line and sorted MAIT cells (See **3.4 Isolation of MAIT cells**) were tested with, following the protocol instructions from the Luminex kit.

Table 3.3. List of cytokines and chemokines tested with the Luminex protocol.

Cytokines and Chemokines			IL-10	GrzA
GM-CSF	CD137	IFN-γ	IL-2	IL-4
IL-13	GrzB	sFAS	MIP-1α	MIP-1β
IL-5	IL-6	sFASL	TNFα	Perforin

3.6 Serum Screen

A serum screen assay was performed to test 11 different serums commercially available. The objective of this screen was to choose the serum that would provide the lowest background and increase the signal for human PBMC samples upon stimulation, specifically on low responder donors. Test serums (**Table 3.4**) were evaluated using 3 different assays (Elispot, Intracellular Staining and Proliferation assay), and compared with a reference serum.

Table 3.4. List of Serums tested in the Serum Screen

Serum Type	Company	Reference Number	EU/mL	Heat Inactivation
1. Human AB Serum	Seralab	SM-612-HS	0.6	no
2. Human AB Serum	Seralab	SM-512-HS	0.3	no
3. Fetal Bovine Serum (FBS)	VWR Seradigm life Science	89510-194	0.2	no
4. Fetal Bovine Serum (FBS)	Biowest	S1810-050	0.2	no
5. Fetal Bovine Serum (FBS)	Hyclone	SV30160.02	0.2	no
6. Fetal Bovine Serum (FBS)	Sigma	F7524-500ML	0.2	no
7. Human AB Serum	Sigma	H6914-100ml	5	no
8. Fetal Bovine Serum (FBS)	Life technologies	1190005	0.5	yes
9. Fetal Bovine Serum (FBS)	Life technologies	1190018	0.5	yes
10. Fetal Bovine Serum (FBS)	Life technologies	10082-147		yes
11. Fetal Bovine Serum (FBS)	Seralab	EU-000F	0.311	no

3.6.1 ELIspot

Enzyme-linked immunospot (ELISPOT) is a high sensitivity assay that provides measurements on the frequency of cytokine-secreting cells at single-cell level (**Figure 3.5**). Upon stimulation with antigens of interest, samples are stimulated on an ELISPOT plate. These are previously coated with capture antibodies against specific cytokines. The capture antibodies will bind to the cell surface and after an incubation period cells are removed, while the secreted molecules of interest remain. These can be detected by adding a cytokine specific detection antibody. The detection antibody is biotinylated and accompanied by a streptavidin-enzyme conjugate, or directly coupled to the enzyme. Finally, a substrate solution is added (BCIP/NBT-plus) is added to the plate, which will reveal a circle of bound cytokines, surrounding the position of each activated cell of interest (spot). Each spot will correspond to an individual cytokine-secreting cell which grants a quantification of the cytokine level within the cellular type analyzed, immediately after secretion.

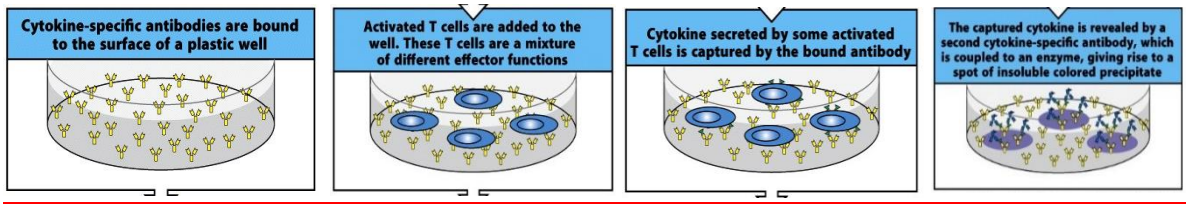

Figure 3.5. Overview of ELIspot technique. Murphy, K., Travers, P., Walport, M., & Janeway, C. (2012). *Janeway's immunobiology* (8th ed.). New York: Garland Science.

In the scope of this project, the ELISPOT assay was performed due to its high sensitivity. The aim of this project is to compare healthy and chronic HBV patients. This assay was used on the serum screen to find the best serum with the lowest background possible, as well as exclude potential false positives results.

Human IFN-γ ELISpot^{PRO} kit (Cat. 3420-2APT-10) was purchased from MABTECH. The kit uses PVDF plates pre-coated with a monoclonal antibody for IFN-γ (Cat. mAb 1-D1K) and one-step detection via alkaline phosphatase (ALP)-conjugated monoclonal antibody (Cat. mAb 7-B6-1-ALP).

BCIP/NBT-plus was used as the substrate for the ALP-conjugated detection mAb. PBMCs were isolated from healthy donors' buffy coats (See **3.1.3 Isolation of Peripheral Blood Mononuclear Cells**). ELIspot plates were blocked with RPMI-1640 medium (Lonza, Cat. 12-702F) supplemented with 1% L-Alanyl-L-glutamine (Sigma, Cat. 68541). The following antigens were added first at appropriate concentrations: PepMix™ CEF pool stimulus (JPT, Cat. PM-CEF-E, at 2µg/mL), consisting of 32 individual peptides, corresponding to a HLA class I-restricted T-cell epitope from CMV, EBV, and Flu viruses (JPT 2µg/mL). A monoclonal antibody against CD-3 (mAb CD3-2/OKT3) was used as a positive control for cell viability and functionality of the assay. DMSO (Sigma - Life Science, Cat. D2650) was used as negative control. From previous studies on Janssen Lab, certain patient samples, were stimulated with individual CEF peptides (**Table 3.5**). All dilutions were made in RPMI-1640 medium (Lonza, Cat. 12-702F) supplemented with 1% L-Alanyl-L-glutamine (Sigma, Cat. 68541). PBS (Sigma, Cat. D8537) was used for washing of the plates. All wells contained 200,000 cells in a volume of 150µL medium. The plates were incubated overnight at 37ºC with 5% CO_2 circulation and wrapped around aluminium foil to avoid evaporation. On the following day, the ELISpot plates were read on CTL ImmunoSpot™ plate reader, according to MABTECH protocol instructions. Counting of spots and quality control was done with CTL ImmunoSpot™. GraphPad Prism 7 was used to visualize the results.

Table 3.5. List of individual CEF peptides used. EBV derived peptides are shown in green (numbers 6 and 19), and CMV derived peptides are shown in blue (numbers 7 and 32).

Index	JPT-#	Sequence	Amount (mg)	Volume DMSO [mL]
6	28167_06	H-GLCTLVAML-OH	1.1	0.55
7	28167_07	H-NLVPMVATV-OH	1.6	0.8
19	28167_19	H-RPPIFIRRL-OH	2.1	1.05
32	28167_32	H-TPRVTGGGAM-OH	2.5	1.25

3.6.2 Intracellular Staining to confirm ELISpot Results

PBMCs were isolated, thawed and counted (See **3.1 Human Samples**). Each sample tested is composed of $1*10^6$ PBMCs per well. Samples were stimulated for 6 hours at 37^0C with PepMix™ CEF pool stimulus, individual CEF peptides (**Table 3.6**) and DMSO as a negative control. The intracellular staining protocol used during this assay was the same as before (See **3.2.1 Flow Cytometry**). However, the antibody panel used was changed to a different set of fluorophores, rendering a slightly different experiment. LIVE/DEAD Fixable Viability Dye 780 (eFluor™) was used as a viability check marker in all samples. All healthy donors' PBMCs were tested for intracellular pro-inflammatory cytokine expression, including IFN-γ, TNF-α and IL-2. PBMC samples were also evaluated for the percentage of canonical T cells, $CD3^+$, $CD4^+$ and $CD8^+$ T cells. Isotypes antibodies for IFN-γ, TNF-α and IL-2 were also used as quality controls. All data acquisition was performed on a BD Bioscience LSR Fortessa™ flow cytometer.

Table 3.6. List of cytokines and isotype markers

Pro-inflammatory Cytokines							
Marker	**Fluorochrome**	**Clone**	**Vendor**	**Catalog**	**AB**	**Volume per Sample (µL)**	**Staining**
CD3	Alexa Fluor 488	RPA-T8	BD bioscience	557694	M	5	Surface
CD4	BUV395	145-2C11-RUO	BD bioscience	564724	M	5	Surface
CD8	BUV786	RPA-T4	BD bioscience	563823	M	5	Surface
IFN-γ	PECF594	B27	BD bioscience	562392	M	5	Intracellular
TNF-α	APC	MAb11	BD bioscience	554514	M	2.5	Intracellular
IL-2	BV421	MQ1-17H12	BD bioscience	564164	R	5	Intracellular
Isotype Control Markers							
Marker	**Fluorochrome**	**Clone**	**Vendor**	**Catalog**	**AB**	**Volume per Sample (µL)**	**Staining**
IgG1	PECF594	x40	BD bioscience	562292	M	5	Intracellular
IgG1	APC	MOPC-21	BD bioscience	554681	M	2.5	Intracellular
IgG2	BV421	R35-95	BD bioscience	562602	R	2.5	Intracellular

3.6.3 Proliferation Assay

PBMCs were thawed and counted. Afterwards, a total amount of 600,000 of PBMCs were placed in each well of a 48 well plate. The cells were stimulated with PepMix™ CEF peptide pool stimulus, individual CEF peptides (**Table 3.5**) and DMSO as a negative control, and incubated at 37^0C with 5% CO_2 for 6 days. All the tested serums (**Table 3.4**) were added to each well for every donor, respectively. After 6 days of stimulation/incubation, cells were stained for LIVE/DEAD viability using the Fixable Viability Dye 780 (eFluor™) and incubated for 30 minutes at 4ºC. All samples were stained for surface markers anti-CD14 (BV510, BD Bioscience, Cat. 563079), anti-CD8 (BV421, BD Bioscience, Cat. 562428), and anti-CD3 (PE-CY7, BD Bioscience, Cat. 557851). Samples were also stained for KI67, a known proliferation marker (FITC, BD Bioscience, Cat. 556026) (**Table 3.7**). At least one sample for each one of the tested donors was stained for an FMO for the proliferation marker Ki-67 as a staining control. After staining, samples were incubated for 30 minutes at 4ºC and the read out performed on a BD Bioscience LSR Fortessa™ flow cytometer.

Table 3.7. Antibodies used in the proliferation assay in serum screen.

Marker	Fluorochrome	Clone	Vendor	Catalog	AB	Volume per Sample (µL)	Staining
CD3	PE-Cy7	SK7	BD	557851	M	2	Surface
CD8	BV421	RPA-T8	BD	562428	M	1	Surface
CD14	BV510	mop9	BD	563079	M	2.5	Surface
KI67	FITC		BD	556026	M ?	10	Surface

Chapter 4 – Results

4.1 Optimizing flow cytometry panel for MAIT cell analysis in human PBMC

The first goal of this project is to determine phenotypically differences in MAIT cells in healthy versus chronical HBV patients. New antibody panels were designed and optimized, and a gating strategy was placed based on fluorescent minus one (FMOs). To achieve this, PBMC populations were stimulated with CD3/CD28 beads and Leukocyte Activation Cocktail (LAC), which were used as positive controls, since they overstimulate and induce unspecific activation of PBMCs and MAIT cells. These experiments were performed on all the flow cytometry antibody panels described in the Materials and Methods section.

Since using LAC, a PHA based, positive stimuli, results were strong but unspecific. It was necessary to find other stimuli that would stimulate and activate MAIT cells with higher specificity. For this purpose, based on other experiments described in the literature[152,199], out-titration experiments were performed using recombinant cytokines IL-12 and IL-18. The activation of MAIT cells as response to IL-12 and Il-18 was measured with the previously described antibody flow panels (**See Materials and Methods**). Both stimuli were tested in several concentrations and at different time points (6 and 24 hours) with the aim of finding optimal or define the sub-optimal concentrations that could increase the activation level of MAIT cells without reaching toxic levels. The addition of Brefeldin A was tested at different time points, without observing any significant differences in MAIT cell activation (data not shown).

We performed two sets of experiments using IL-12 and IL-18 as single stimuli. First, we tested 3 different concentrations for each stimulus: IL-12 was evaluated at 50, 10 and 2ng/mL, and IL-18 at 100, 50 and 10ng/mL. There was no dose depended response observed (data not shown). This could indicate that, at higher stimuli concentrations, the percentage of MAIT cells activated reaches a maximum level remaining equal. Hence, even if the concentration of the stimuli is increased, the percentage of MAIT cells that are activated remains the same. Lower concentrations of IL-12 and IL-18 were tested to identify a sub-optimal concentration for both single stimuli. The concentrations used were 1, 0.25 and 0.0625ng/mL for IL-12 single stimulation and 5, 1 and 0.5ng/mL for IL-18 stimuli. After optimizing the panels for the evaluation of MAIT cells in human PBMCs, we decided to use three different concentrations for both IL-12 (10, 1 and 0.25ng/mL) and IL-18 stimuli (50, 10, 1ng/mL).

4.2 MAIT cell phenotyping (% population in PBMC)

4.2.1 Surface markers (CD4/CD8 and CD56) within the MAIT cell population in donor PBMC

PBMCs from each donor were stimulated with different doses of IL-12 and IL-18. PBMCs were sained with different cellular markers to identify MAIT cells. MAIT cells were gated and their frequency measured for the percentage of $CD4^+$, $CD8^+$ and $CD56^+$ MAIT cells. As shown in **Figure 4.1**, the percentage of MAIT cells was evaluated within the $CD3^+$ T cell population for both sample groups (healthy *versus* chronic HBV patients). The results show that there is no clear difference between healthy controls and HBV chronically infected patients. After stimulation, the same frequency was observed independently of the stimuli concentrations of either IL-12 or IL-18. We observed a clear donor variability in both groups, which might influence our comparative analysis. Still, our results are consistent to what is documented on the literature, 1 to 10% of PBMCs population are indeed MAIT cells. The total number of MAIT cells was compared with the unstimulated control samples obtaining the same results, and equal to the unstimulated control samples (**Figure 4.1**).

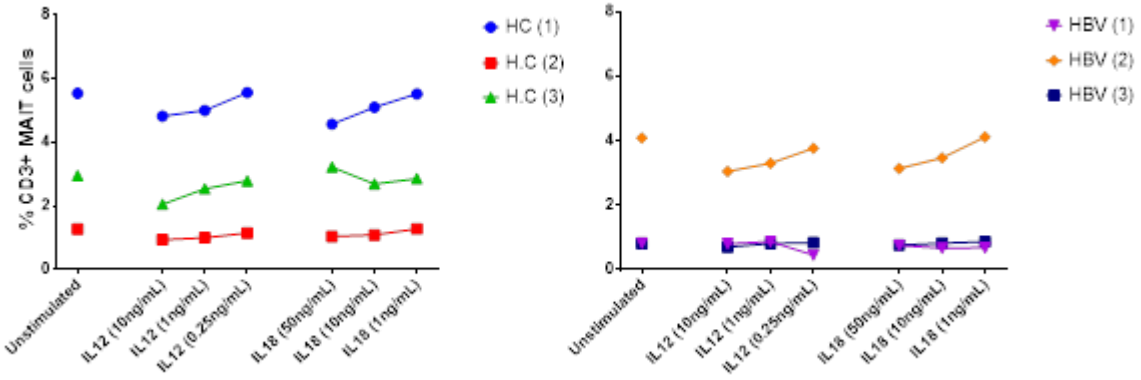

Figure 4.1 Quantification of MAIT cells within the CD3$^+$ T cell population (indicated in % from total CD3$^+$ T cells). MAIT cells stimulation for 24 hours with IL-12 and IL-18 recombinant innate cytokines at different concentrations: IL-12 (0.25/1 and 10ng/mL) and IL-18 (1/10 and 50ng/mL). MAIT cells from 3 Healthy Control Patients and 3 Chronic HBV patients were tested

The percentage of CD4+ and CD8+ MAIT cells in healthy and chronic patients were evaluated (**Figure 4.2**). Healthy patients showed similar frequencies of CD4$^+$ MAIT cells independently of the stimuli used, ranging from 0 to 10%. Donor HC3 showed higher levels upon stimulation with IL-18 at 50ng/mL, compared to the other two donors. On the contrary, when evaluating the chronic HBV patients, higher variation on the frequency CD4+MAIT cell was observed. Two of the donors, HBV1 and HBV3, seemed to contain higher levels of these cells compared to HBV 2. Additionally, when healthy and chronic HBV donors were compared, the percentage of CD4$^+$ MAIT cells was around three times higher in the latter independently of the stimuli concentration.

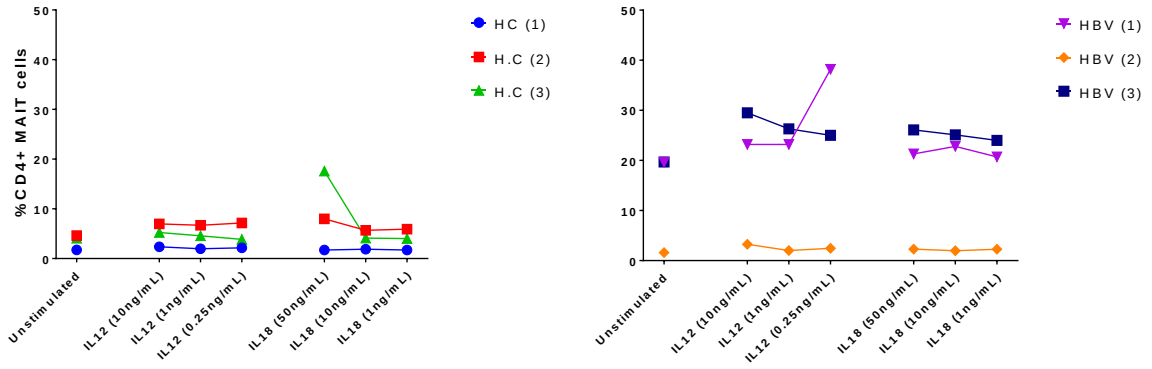

Figure 4.2 Quantification of CD4$^+$ within the MAIT cell population (indicated in % from total MAIT cells). MAIT cells stimulation for 24 hours with IL-12 and IL-18 recombinant innate cytokines at different concentrations: IL-12 (0.25/1 and 10ng/mL) and IL-18 (1/10 and 50ng/mL). MAIT cells from 3 Healthy Control Patients and 3 Chronic HBV patients were tested

Next, the percentage of CD8$^+$ MAIT cells (**Figure 4.3**) was analysed. It was clear that no change existed among the healthy patients after stimulating conditions and that the majority of MAIT cells in the periphery were CD8$^+$ effector cells. The percentage of CD8+ MAIT cells for all donors ranged between 80 and 100%. On the contrary, the chronic HBV donors presented different frequencies of CD8+ MAIT cells. HBV 2 donor showed similar percentage of CD8+ MAIT cells to that observed on healthy patients. However, donors HBV1 and HBV3 showed lower population

frequency of CD8+ MAIT cells, around 60%. No increase in MAIT cell frequency were expected, due to the limited time of stimulation, 24 hours.

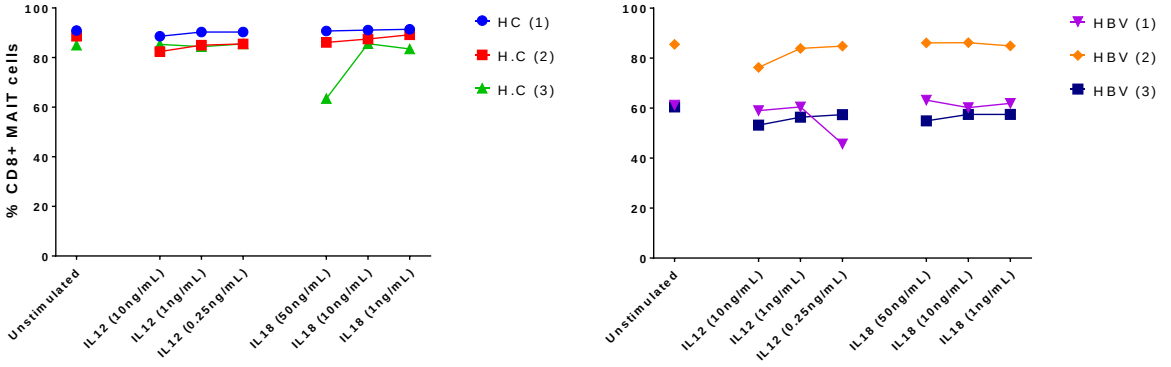

Figure 4.3 Quantification of CD8$^+$ within the MAIT cell population (indicated in % from total MAIT cells). MAIT cells stimulation for 24 hours with IL-12 and IL-18 recombinant innate cytokines at different concentrations: IL-12 (0.25/1 and 10ng/mL) and IL-18 (1/10 and 50ng/mL). MAIT cells from 3 Healthy Control Patients and 3 Chronic HBV patients were tested

According to the literature, there is a MAIT cell sub-population that is positive for CD56 [152]. Thus, the data was analysed for CD56$^+$ MAIT cells. In **Figure 4.4**, the results for the percentage of MAIT cells that are CD56$^+$ are shown. When comparing both groups, the levels of CD56$^+$ MAIT cells were similar between healthy controls and chronic HBV patients. One large deviation in donor HC3 was observed. when stimulated with IL-18 at the highest concentration of 50ng/mL almost 50% of MAIT cells were CD56$^+$. Also, the unstimulated CD56$^+$ MAIT cells samples show a baseline of 20% and in the lower IL-18 stimulation the results remain similar.

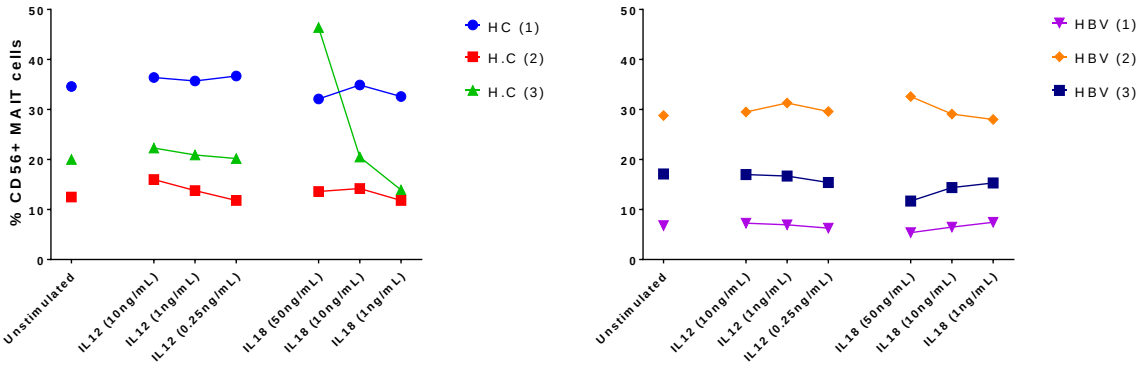

Figure 4.4 Quantification of CD56$^+$ within the MAIT cell population (indicated in % from total MAIT cells). MAIT cells stimulation for 24 hours with IL-12 and IL-18 recombinant innate cytokines at different concentrations: IL-12 (0.25/1 and 10ng/mL) and IL-18 (1/10 and 50ng/mL). MAIT cells from 3 Healthy Control Patients and 3 Chronic HBV patients were tested

4.3 *Ex vivo* MAIT cell activation

4.3.1 Checkpoint Inhibitors

MAIT cell population was evaluated for its checkpoint inhibitory marker expression level. The following checkpoint inhibitors assessed: PD-1, TIM3 and LAG3, as previously mentioned (See **3.2.1 Flow Cytometry**). The results obtained for these markers are illustrated on **Figure 4.5**, **Figure 4.6** and **Figure 4.7**. MAIT cells from chronic HBV patients showed higher PD-1 expression levels than those observed for HC donors (**Figure 4.5**). Indeed, even if we take into consideration the high

background levels present in the unstimulated samples, and the variability among donors upon IL-12 and IL-18 stimulation, all the chronic HBV donors expressed PD-1 in more than 55% of the cells. On the contrary, only HC2 seemed to be positively activated amongst the HCs, with around 60% of the cells expressing PD-1 when stimulated with IL-12 at 10ng/mL. Also, it is important to mention that despite the high background levels, all donors responded in a positive manner, demonstrating a dose-response curve upon IL-12 stimulation. However, when analysing the results for IL-18 stimulation, only donor HC2 responded to the stimulus, showing high percentage of PD-1^{+} MAIT cells at a concentration of 50ng/mL compared to baseline levels. As for the chronic HBV donors, both HBV2 and HBV3 increased PD-1 expression in a dose-dependent manner. Also, we observed that HBV3 reaches its PD-1 expression peak at 10ng/mL of IL-18 and, when the concentration of this stimulus increases, this donor decreased its PD-1^{+} expression levels. The differences among HC and HBV donors might happen due to presence of the PD-1 marker itself, hence in a case where there is no apparent infection, PD-1 can function as an activator, and upon a chronic infection it can have an inhibitory effect.

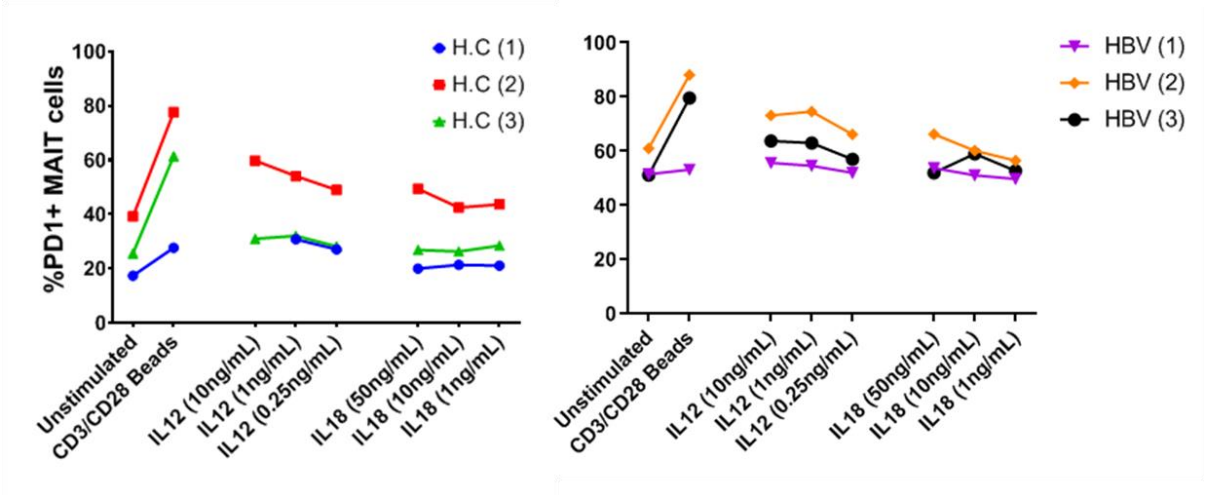

Figure 4.5 Quantification of PD-1^{+} MAIT cells within the PBMC population (indicated in % from total MAIT cells). MAIT cells stimulation for 24 hours with IL-12 and IL-18 recombinant innate cytokines at different concentrations: IL-12 (0.25/1 and 10ng/mL) and IL-18 (1/10 and 50ng/mL) and CD3/CD28 Beads as positive control. MAIT cells from 3 Healthy Control Patients and 3 Chronic HBV patients were tested

The expression of another MAIT cells checkpoint inhibitor, TIM3 was also evaluated and compared between healthy and chronic HBV donors (**Figure 4.6**). The results shown in figure 4.6 demonstrate a high variability among donors of each group, but still suggest a positive response from the cells upon stimulation. On the left side, HC2 showed a dose-response activation upon stimulation with IL-12, and the highest values of TIM3 expression, with more than 30% of MAIT cells expressing this checkpoint inhibitor at a stimulus concentration of 10ng/mL. As for HC1 and HC3 donors, although they seem to have responded positively when stimulated with IL-12, their results varied. HC1 already displayed its maximum TIM3 expression at 0.25ng/mL, while HC3 seemed to increase the level of expression for this marker only at 1ng/mL. In addition, when stimulated with IL-18, all HCs responded in a positive manner. However, HC1 and HC2 reached the maximum percentage of MAIT cells expressing TIM3 at a concentration of 1ng/mL, whereas HC3 showed a similar effect to that observed upon IL-12 stimulation, reaching the maximum level of expression (10% of the cells were positive for this marker) at 10ng/mL of IL-18. All the results obtained were compared with the unstimulated samples, which give an input on the background expression levels.

On figure 4.6 right graph, it is shown that CHB patients have slightly higher baseline TIM-3 expression than HC, which is not as evident as in PD-1 expression. Two of the donors, HBV1 and

HBV3, stand out in the group for both stimuli, since their response was not dependent on the concentration used for each stimulus, and the concentration that led to higher expression levels of TIM3 was not the same for both donors. HBV1 showed little response to the lowest concentration used. However, when increasing concentrations (to 1 ng/ml for IL-12 and to 10 ng/ml for IL-18), this donor expressed 3 times more TIM3, in comparison with the lowest concentration. We also noticed that, after reaching this peak of TIM3 expression (30% of the cells expressing the marker) for both stimuli, expression levels decreased with increasing concentrations, suggesting that above a certain concentration of stimulus cells might die or be unable to further express this marker. The same was observed for donor HBV3. However, in this case, the maximum levels of expression were seen already at the lowest concentration of both stimuli, 0.25 and 1ng/mL for IL-12 and IL-18, respectively. Here, the maximum percentage of MAIT cells expressing TIM3 was approximately 35%. Regarding HBV2 donor, MAIT cells seemed to be expressing TIM3 when stimulated with IL-12, but no differences in their response were seen between different concentrations of stimulus. However, when analysing the same donor upon IL-18 stimulation, HBV2 does not upregulate TIM3 as much as HBV chronic, but seem to have similar levels to healthy volunteers.

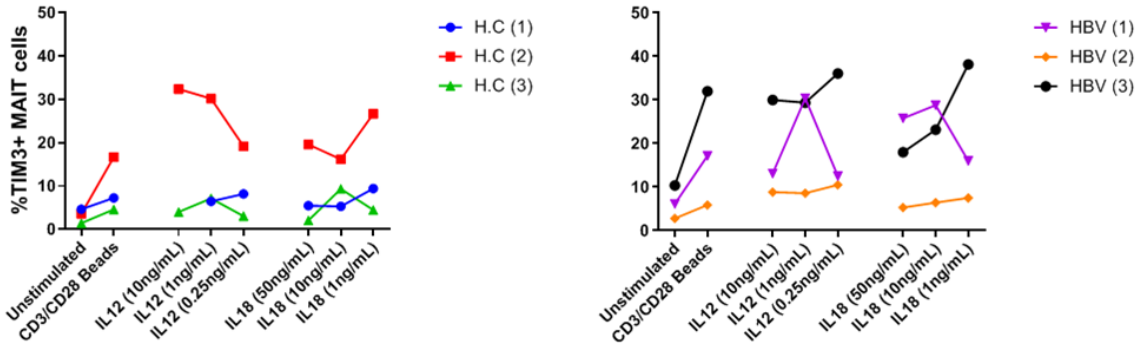

Figure 4.6 Quantification of TIM3$^+$ MAIT cells within the PBMC population (indicated in % from total MAIT cells). MAIT cells stimulation for 24 hours with IL-12 and IL-18 recombinant innate cytokines at different concentrations: IL-12 (0.25/1 and 10ng/mL) and IL-18 (1/10 and 50ng/mL) and CD3/CD28 Beads as positive control. MAIT cells from 3 Healthy Control Patients and 3 Chronic HBV patients were tested

The percentage of MAIT cells expressing the checkpoint inhibitor LAG3 was also assessed. The first thing we noticed when analysing the results displayed in **Figure 4.7**. was that there is a much lower percentage of MAIT cells expressing LAG3, in comparison to the previously evaluated markers, PD-1 and TIM3. Also, HC donors did not show a clear response to any of the used stimuli. Therefore, no significant increase in the expression of the LAG3 marker was observed for these donors, independently of the concentration of stimuli used, and in comparison, to the unstimulated samples. Nevertheless, upon stimulation with IL-12 at a concentration of 10ng/mL, HC2 demonstrated an increase in the percentage of MAIT cells expressing LAG3, reaching almost 2%. On the other hand, all the healthy control appeared to be unresponsive to IL-18 stimulation. Additionally, the response and expression of the inhibitory marker LAG3 was also assessed in chronic HBV patients. The variability among donors was evident, as well as the lack of response both HBV2 and HBV3 upon stimulation with IL-18. Nonetheless, with regards to IL-12 stimulation, the percentage of MAIT cells expressing LAG3 increased with increasing concentrations of the IL-12 stimulus. Despite this, all donors responded differently. MAIT cells from this donor HBV1 reached peak expression of LAG3 at 1ng/mL (around 8%). At the maximum concentration tested of IL-12, a drop in LAG3 expression levels was observed. HBV2 donor showed a dose-response to IL-12 stimulation in the first two concentrations of stimulus, but once it reached the maximum expression levels of LAG3 (at

1ng/mL of IL-12), the percentage of MAIT cells expressing LAG3 (around 1.5%) remained equal to that observed at the highest concentrations of IL-12suggesting that this donor reached a plateau phase. Finally, HBV3 donor positively responded to stimulation with different concentrations of IL-12, increasing the percentage of MAIT cells expressing LAG3 in a dose-dependent manner. This donor reached the maximum levels of expression at 10ng/mL of IL-12 (approximately 6% of MAIT cells expressed the marker). As stated before, only HBV1 was shown to be responsive to IL-18 stimulation, and the results were similar to the ones obtained with IL-12 stimulation. HBV1 reached the maximum level of expression of LAG3 at a concentration of 10ng/mL, whereas the lowest concentration used showed the same result as the unstimulated sample. MAIT cells from CHB patients have 3-4 fold higher maximum LAG-3 expression than HC, and peaking already at 1ug/mL IL-12/18 stimuli, suggesting a much higher sensitivity to checkpoint upregulation and possibliy entering an exhaustive state

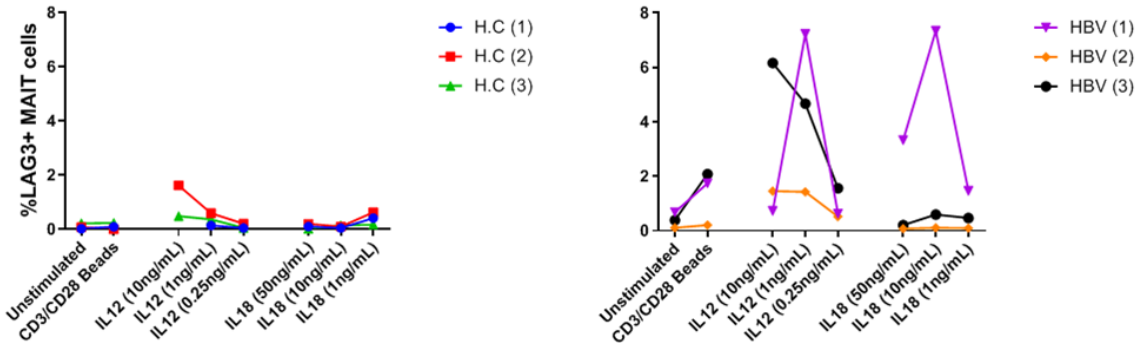

Figure 4.7 Quantification of LAG3$^+$ MAIT cells within the PBMC population (indicated in % from total MAIT cells). MAIT cells stimulation for 24 hours with IL-12 and IL-18 recombinant innate cytokines at different concentrations: IL-12 (0.25/1 and 10ng/mL) and IL-18 (1/10 and 50ng/mL) and CD3/CD28 Beads as positive control. MAIT cells from 3 Healthy Control Patients and 3 Chronic HBV patients were tested

4.3.2 Activation Markers

Are MAIT cells still able to be activated in chronic HBV? To answer this question, the expression level of different activation markers - CD25, CD38, CD69 and HLA-DR - were measured on MAIT cells. **Figure 4.8** shows the results for CD25$^+$ MAIT cells. A clear difference was observed between HC and chronic HBV patients. Chronic HBV patients demonstrated higher expression of the activation marker CD25. The healthy patients tested do not show a dose-response for CD25 activation after 24 hours stimulation. The same activation pattern was expected from the chronic HBV patients. Despite reacting in a similar manner upon stimulation with IL-12 and IL-18 at different concentrations, donor HBV3 presented a different activation response. This donor displayed a steep dose-response in the number of CD25$^+$ MAIT cells upon stimulation with different concentrations of IL-18. At this concentration, approximately 30% of the cells expressed CD25. Regarding HC donors, HC1 and 3 showed almost no CD25$^+$ MAIT cells. On the opposite, HC2 contains MAIT cells that express CD25, maybe responding in a dose-dependent manner for the IL-12 stimulus. However, it is not easy to perceive, due to the exclusion of some data points (HC2 and HBV3) for the entire antibody panel. The values excluded lacked viability percentages above 70%, minimum required to be used in immunological assays.

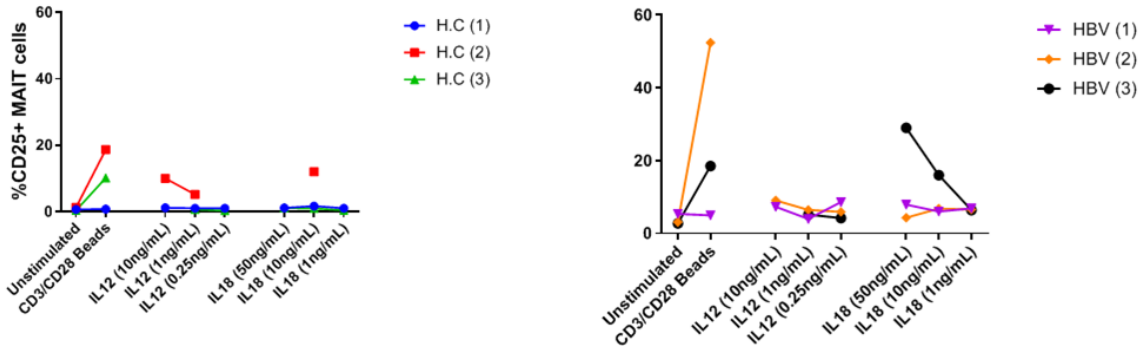

Figure 4.8. Quantification of CD25⁺ MAIT cells within the PBMC population (indicated in % from total MAIT cells). MAIT cells stimulation for 24 hours with IL-12 and IL-18 recombinant innate cytokines at different concentrations: IL-12 (0.25/1 and 10ng/mL) and IL-18 (1/10 and 50ng/mL) and CD3/CD28 Beads as positive control. MAIT cells from 3 Healthy Control Patients and 3 Chronic HBV patients were tested

CD38, a well-established late activation marker, was also tested (**Figure 4.9**). When comparing HC *versus* chronic HBV donors, the first observation was the difference in the frequency of CD38⁺ MAIT cells. HBV patients clearly presented higher CD38 expression levels, even though there was high variability between the donors. When stimulated by different concentrations of IL-12 and IL-18, HBV1 exhibited no changes in CD38 expression levels. Upon stimulation with IL-12, HBV2 responded in a dose-response manner, showing the highest CD38 expression levels at 10ng/ml of stimulus. At this concentration, around 60% of MAIT cells were positive for CD38 expression. Though this donor still demonstrated CD38 expression upon IL-18 stimulation, it seems that at the two highest concentrations there was no change in CD38 expression levels perhaps due to a saturation effect. Donor HBV3 does not show a dose-response increase of CD38 after stimulation with IL-12, which might be influenced by the exclusion of the values for the highest IL-12 concentration used. However, after stimulation with IL-18, this donor responded in a dose-depended manner, reaching the highest CD38 expression levels at 50ng/mL of IL-18 (~30% of CD38+ MAIT cells). Contrarily, HC donors did not show an increase in CD38 expression after stimulation with any of the stimuli. For instance, donors HC1 and 3 displayed no change in CD38 expression levels, when compared to the unstimulated samples. Nevertheless, HC2 donor reacted positively upon stimulation with IL-12 at 10ng/mL, while IL-18 seemed to induce effect at a concentration of 10ng/mL.

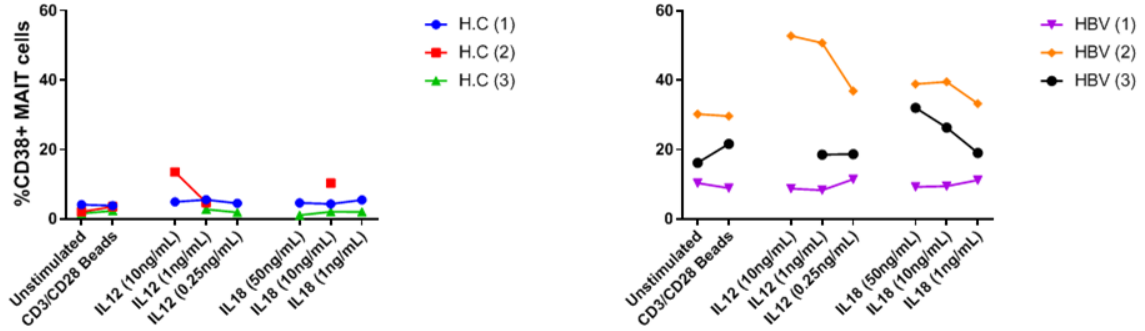

Figure 4.9. Quantification of CD38⁺ MAIT cells within the PBMC population (indicated in % from total MAIT cells). MAIT cells stimulation for 24 hours with IL-12 and IL-18 recombinant innate cytokines at different concentrations: IL-12 (0.25/1 and 10ng/mL) and IL-18 (1/10 and 50ng/mL) and CD3/CD28 Beads as positive control. MAIT cells from 3 Healthy Control Patients and 3 Chronic HBV patients were tested

Simultaneously, early activation markers CD69 and HLA-DR were investigated on MAIT cells (**Figure 4.10** and **Figure 4.11**). The first observations, MAIT cells showed higher background levels in the unstimulated samples. After stimulation, variability among donors was observed.

As shown in **Figure 4.10**, all the HC donors reacted to IL-12 stimulation and demonstrated a dose-response curve to this stimulus. A dose-response pattern was shown after IL-18 stimulation in 2 out of 3 donors. Donor HC2 only shows one value point for the IL-18 stimulation, while the other two points were removed due to a low viability frequency (< 70%). HC1 showed the highest CD69 expression levels among the healthy controls, in which around 80% of MAIT cells expressed CD69, even though we observed a slight decrease on CD69 expression levels at the highest concentration of IL-18 stimulus. HC3 still responded to stimulation with IL-18 but at lower levels, only 60% of MAIT cells expressed CD69. The percentage of MAIT cells expressing CD69 in HC2 can be considered null, since the background in the unstimulated sample was high and comparable to after IL-18 stimulation, although only one value is shown in the graph (stimulation at 10 ng/mL of IL-18). On the other hand, HBV donors showed a few differences. For example, donor HBV2 showed a large deviation, compared to the other two chronic HBV donors in expression of CD69 by MAIT cells. However, donors HBV1 and HBV3 were the ones that responded to stimulation in a dose-response manner, even though one value point was excluded for donor HBV3. Also, when comparing the background levels for healthy and chronic HBV patients, 2 out of 3 HBV donors demonstrated lower background levels than the healthy controls. The highest percentage of MAIT cells expressing CD69 was observed after stimulation with 1ng/mL for IL-12 and 10ng/mL for IL-18. With regards to HBV2 donor, despite demonstrating the highest percentage of MAIT cells expressing CD69 (almost 100%), this donor did not show a dose-depended response after stimuli due to the high background levels. Finally, donor HBV3 only reacted to stimulation with 10ng/mL of IL-12. This donor did not react to all the other stimuli concentration tested, since CD69 expression levels were below the background levels.

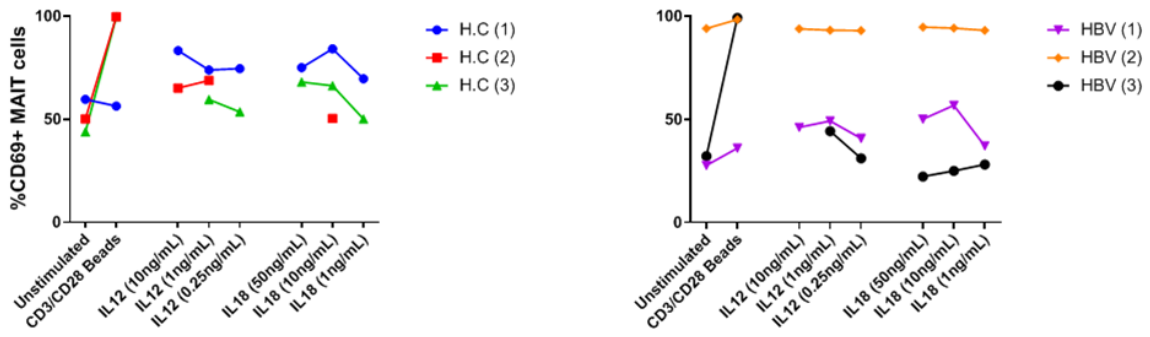

Figure 4.10. Quantification of CD69⁺ MAIT cells within the PBMC population (indicated in % from total MAIT cells). MAIT cells stimulation for 24 hours with IL-12 and IL-18 recombinant innate cytokines at different concentrations: IL-12 (0.25/1 and 10ng/mL) and IL-18 (1/10 and 50ng/mL) and CD3/CD28 Beads as positive control. MAIT cells from 3 Healthy Control Patients and 3 Chronic HBV patients were tested

In **Figure 4.11** is showed the results for the percentage of MAIT cells expressing the HLA-DR activation marker in healthy and chronic HBV donors. Within the HC patients tested, the HLA-DR expression levels seemed to be similar. Still, healthy donors reacted positively after stimulation with both IL-12 and IL-18, since levels of HLA-DR expression were higher than the background levels. When looking at each stimulus separately, all HCs show to have a dose-response after IL-12 stimulation with a maximum HLA-DR expression at 10ng/mL (35 to 55% of MAIT cells). On the

other hand, when stimulated with IL-18, the expression levels of HLA-DR remained similar between all HC individuals, independently of the concentration of stimuli used, which is around 20 to 40%.

Regarding the chronic HBV donors, variability among donors and the background levels were higher than healthy controls. HBV1 showed no activation when stimulated with IL-12 and IL-18 at different concentrations. In contrast, HBV2 showed a dose-response for both stimuli. Upon IL-18 stimulation, this donor response was identical to donor HBV3 with a maximum of 60% expression at 50ng/mL of IL-18. When the PBMC were stimulated with IL-12, HBV3 exhibited an increase in HLA-DR expression, with almost 80% of the MAIT cells expressing this marker at the highest concentration for IL-12, 10ng/mL. Even considering the variation within the donors, both healthy and chronic HBV donors responded similarly to IL-12 and IL-18.

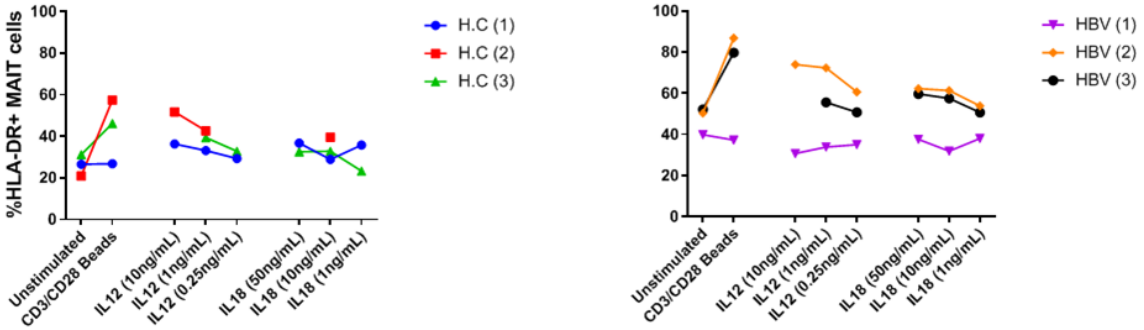

Figure 4.11. Quantification of HLA-DR$^+$ MAIT cells within the PBMC population (indicated in % from total MAIT cells). MAIT cells stimulation for 24 hours with IL-12 and IL-18 recombinant innate cytokines at different concentrations: IL-12 (0.25/1 and 10ng/mL) and IL-18 (1/10 and 50ng/mL) and CD3/CD28 Beads as positive control. MAIT cells from 3 Healthy Control Patients and 3 Chronic HBV patients were tested

4.3.3 Pro-Inflammatory Cytokines

To investigate whether MAIT cells are still functional in chronic hepatitis B patient's, cytokine production (Il-2, IFN-g, TNF-a) after stimulation of MAIT cells with IL12 or IL-18 was measured (See **3.2.1 Flow Cytometry**). **Figure 4.12**, **Figure 4.13** and **Figure 4.14** show the levels of expression of pro-inflammatory cytokines IFNγ, TNFα and IL-2. **Figure 4.12** compares % of MAIT cells that express IFNγ in healthy donors (left) and HBV donors (right). No apparent reduction of IFNγ production in MAIT cells correlated to chronic HBV infection was observed. Despite showing higher expression levels of IFNγ, upon IL-18 stimulation only 1 out of 3 donors in each group (HC2 and HBV2) show up to 15% of IFNγ$^+$ MAIT cells, while the remaining donors for each group did not seem to respond to the stimuli. These donors demonstrated a clear dose-response upon IL-12 stimulation, showing almost 16 times more IFNγ expression at the highest concentration (10ng/mL), in comparison to the unstimulated control samples. HBV3 demonstrated the highest level of IFNγ expression upon IL-12 stimulation at 1ng/mL, while increasing the concentration of this stimulus led to a saturation point of IFNγ expression by MAIT cells.

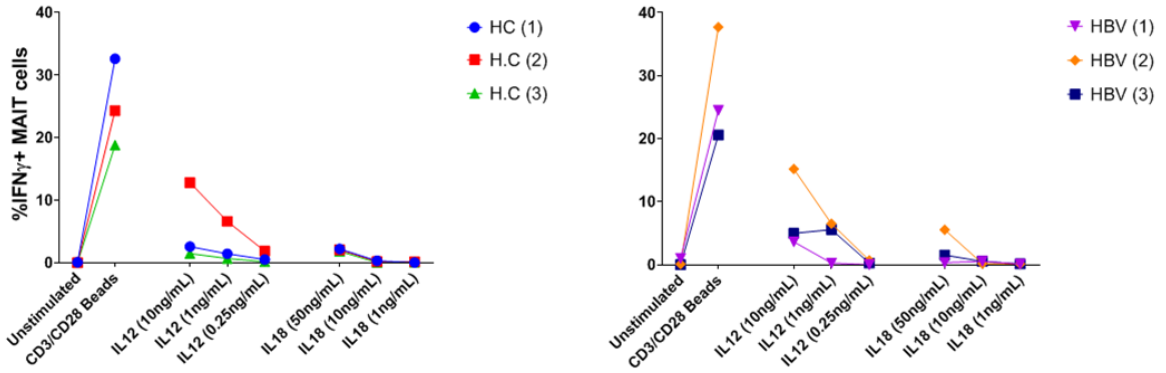

Figure 4.12. Quantification of IFNγ $^{+}$ MAIT cells within the PBMC population (indicated in % from total MAIT cells). MAIT cells stimulation for 24 hours with IL-12 and IL-18 recombinant innate cytokines at different concentrations: IL-12 (0.25/1 and 10ng/mL) and IL-18 (1/10 and 50ng/mL) and CD3/CD28 Beads as positive control. MAIT cells from 3 Healthy Control Patients and 3 Chronic HBV patients were tested

The percentage of TNFα and IL-2 cytokine expression was also evaluated within the MAIT cell population (**Figure 4.13** and **Figure 4.14**). As seen with IFNγ, the results between HCs and chronic HBV patients the expression levels of TNFα and IL-2 were similar

In general, all donors displayed MAIT cells expressing TNFα ranging from 0 and 1% (**Figure 4.13**) and lower than the results observed for the expression of IFNγ. MAIT cells from sample HBV1 were observed to have an unusual TNFα expression, with background values at 1.6% and much lower TNFα after stimulation with IL-12/18 except at 10ng/mL IL-12. The high background of TNFα in sample HBV1 makes it difficult to deduce changes in TNFa functionality of this sample.

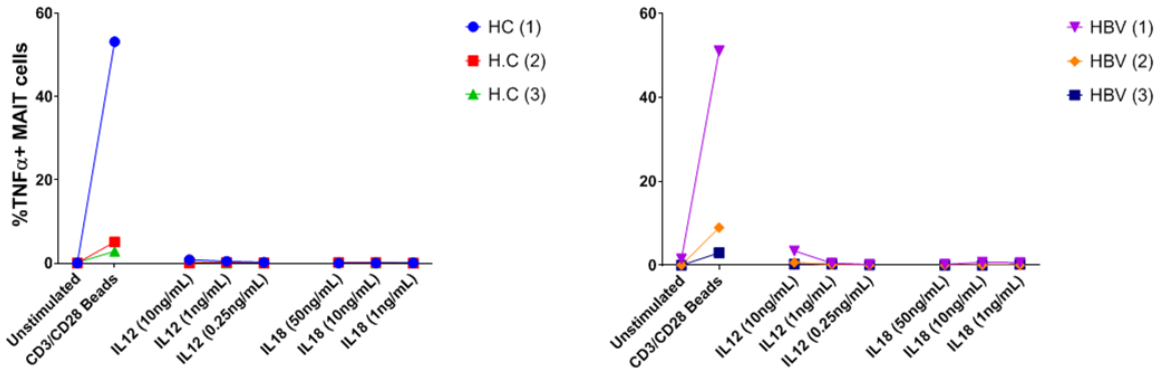

Figure 4.13. Quantification of TNFα^{+} MAIT cells within the PBMC population (indicated in % from total MAIT cells). MAIT cells stimulation for 24 hours with IL-12 and IL-18 recombinant innate cytokines at different concentrations: IL-12 (0.25/1 and 10ng/mL) and IL-18 (1/10 and 50ng/mL) and CD3/CD28 Beads as positive control. MAIT cells from 3 Healthy Control Patients and 3 Chronic HBV patients were tested

IL-2 cytokine expression levels by MAIT cells was also measured (**Figure 4.14**). As illustrated below, there were no major differences in the IL-2 expression pattern between HC and chronic HBV patients. Also, the levels of IL-2 expression are so diminished that the production of this cytokine by MAIT cells was almost inexistent. Furthermore, the different concentrations used for each of the stimulus did not seem to increase or decrease the number of MAIT cells that express IL-2. Despite this, it is important to mention that, within the HC donors, HC3 demonstrated a positive reaction upon stimulation with IL-18 at 50ng/mL, whereas sample CHB donor HBV1 shows a larger

IL-2 population in the control conditions, than in stimulated conditions, making functional evaluation of IL-2 response very difficult.

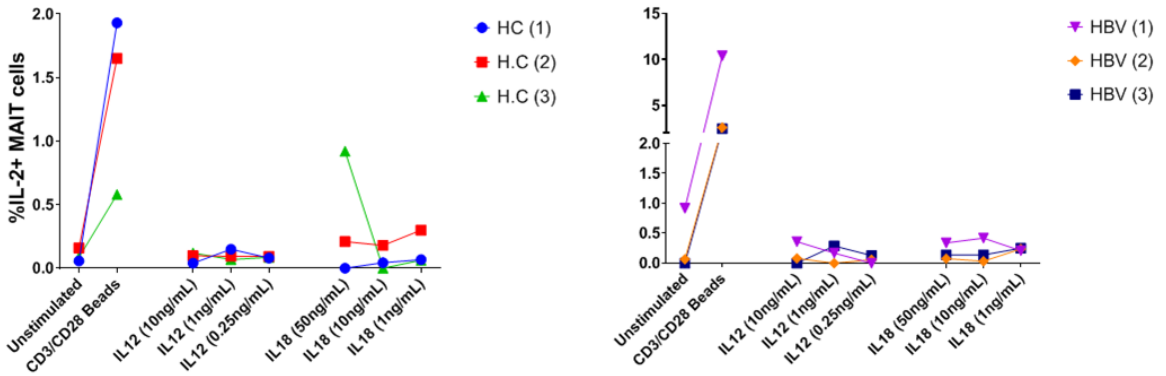

Figure 4.14. Quantification of IL-2^{+} MAIT cells within the PBMC population (indicated in % from total MAIT cells). MAIT cells stimulation for 24 hours with IL-12 and IL-18 recombinant innate cytokines at different concentrations: IL-12 (0.25/1 and 10ng/mL) and IL-18 (1/10 and 50ng/mL) and CD3/CD28 Beads as positive control. MAIT cells from 3 Healthy Control Patients and 3 Chronic HBV patients were tested

4.4 MAIT cell isolation with beads

While evaluating the expression of different cellular markers on MAIT cells, we tested two different methods to isolate these cells within a PBMC population: Bead Isolation and Cell Sorting (See Materials and Methods). The isolation of MAIT cells was necessary to carry out functional assays together with a lymphoblast cell line - HMy2.CIR [C1R, HMy2.C1R]. This allowed us to assess MAIT cells ability to be activated in the presence of an antigen presenting cell line, and address whether MAIT cells express similar or different cytokines and chemokines as the ones previously described (See **Introduction**). First, we followed a beads sorting protocol from Miltenyi Biotech, using two specific known markers for MAIT cells, TCRVα7.2 and CD161, to evaluate their viability after the isolation process. In figure ZX, some of the steps of this isolation are depicted. Based on these results, we concluded that the experimental procedure did not work completely. Before sorting MAIT cells, more than 90% of the PBMC population was viable. However, when assessing the percentage of MAIT cells double positive for TCRVα7.2 and CD161, we observed that almost none (3.74%) of the MAIT cells isolated were viable, meaning that cell death occurred along the procedure. Thus, we decided to include an extra step on the experimental procedure using a dead removal kit (See **3.4.1 Isolation of MAIT cells with Microbeads Separation Kit**). The aim was to remove the highest percentage possible of dead cells that could influence the viability of isolated MAIT cells. However, even with this extra step, the percentage of viable MAIT cells within the PBMC population before bead sorting (4.74%) was much higher than after sorting, whereas the percentage of viable MAIT cells double positive for TCRVα7.2 and CD161 was almost inexistent within the 4.74%, representing the total MAIT cell population within PBMCs.

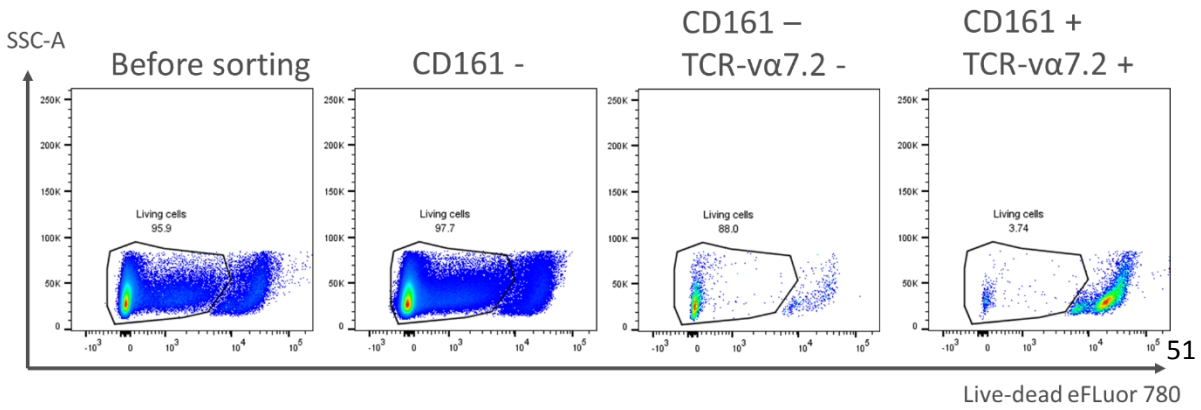

Figure 4.15. Overview of bead sorting of MAIT cells using two different beads marker: TCRVα7.2 and CD161

The next step was to use a cell sorter (See **3.4.2 FACS Cell-Sorting**) instead of beads isolation protocols. **Figure 4.16** illustrates an example of MAIT cells sorting within a PBMC population obtained from a healthy donor. This procedure provided better results, as shown by the purity percentage of MAIT cells after sorting (99.3%). The same monoclonal antibodies used in the bead isolation experiment were applied in this technique, as these are known specific cellular markers used in the identification of MAIT cells. **Figure 4.16** represents the viability percentage of the initial PBMC population, as well as the purity percentage of MAIT cell within that population.

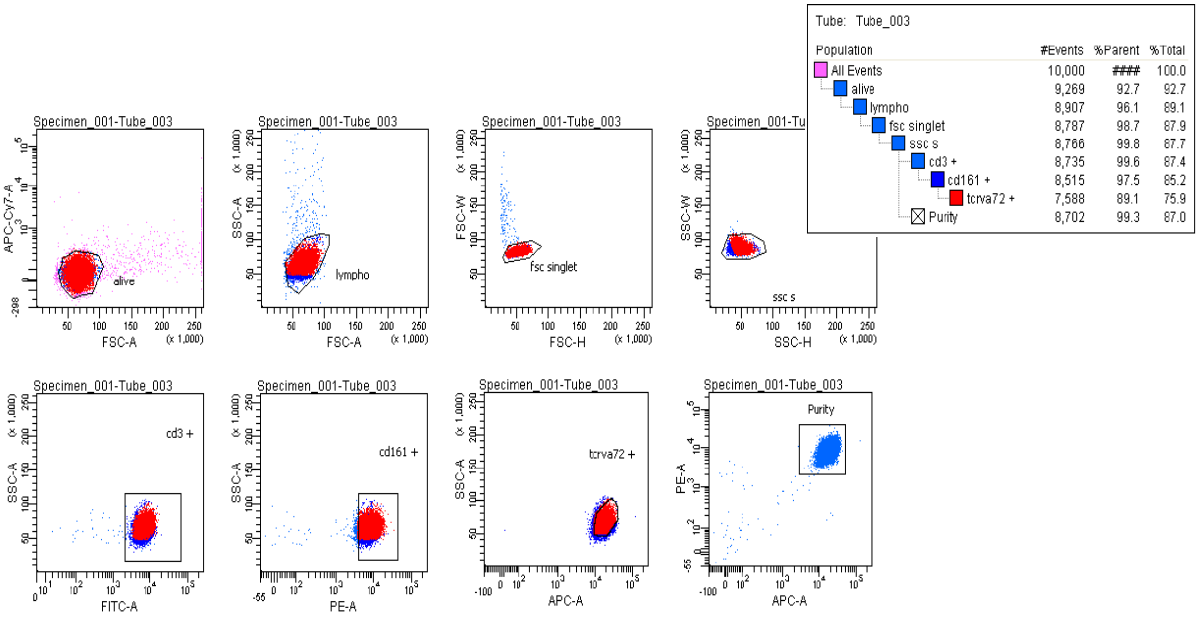

Figure 4.16. Overview of Cell Sorting of MAIT cells from PBMC population from a healthy donor

Sorted MAIT cells were tested in a functional assay together with HMy2.CIR [C1R, HMy2] cell line. Cells were stimulated and evaluated using Luminex technology as previously mentioned (See **3.5.4 Luminex**). Unfortunately, the cytokine assay did not pass qualification, since MAIT cells showed activation patterns even before being co-cultured with the cell line or stimulated (data not shown). Also, it seemed that the viability of MAIT cells was low when these cells were sorted and stimulated for a certain period of time. This might be one reason for the inability to observe expression of cytokines/chemokine upon stimulation on Luminex.

4.5 Serum screening for PBMC culture media

A serum screen for PBMC culture media was performed using three different assays (ELIspot, Proliferation assay and ICS), in which 11 different serums coupled to healthy donors were tested (See **3.6 Serum Screen**).

4.5.1 ELIspot

Six healthy donors were assessed for their response, translated by the number of spot forming cells, upon stimulation with CEF pool peptides, individual CEF peptides and DMSO (See **3.6.1 ELIspot**). In **Figure 4.17**, the results obtained for the ELISPOT assay are shown. Human donors can be classified as low, intermediate or high responders, depending on the scientific assay they are tested for. Since the overall objective of this project was to compare healthy patients against chronic HBV patients, the latter known to have week responses when tested in assays such as ELISPOT, ICS and Proliferation assays, in this experiment we used intermediate and low responder donors. DMSO was used as a negative control and we confirmed this control worked when compared to the results from

the positive stimulation with CEF pool peptides or with individual peptides, as shown in **Figure 4.17** and in **Figure 4.18**. Serum 10 was also used as control, since this serum was used for all the immunological assays performed *in house.* Also, due to the high sensitivity of the ELISPOT assay, a cut-off line was placed on 50 spots forming cells (SFCs) per million PBMCs, whereas values below this threshold were considered not accurate to take conclusions in the context of this technique.

When stimulated with a pool of 32 CEF peptides, screen donor 1611 (shown in **Figure 4.17.A**) responded in a similar manner to all the serums tested. There was small variability with this donor amongst the 11 serums tested, and results ranged between 500 and 1000 SFCs per million PBMCs. Still, serums 1, 2, 4, 6, 7, 8 and 10 exhibited the best results among all the serums. Notably, serum 1 reached approximately 1000 SFCs per million PBMCs. The same pattern can be observed for screen donors represented by figures **Figure 4.17.B, C and D**, where there was not a substantial difference among the serums tested. Although the differences are not obvious between distinct serums, it is possible to highlight some serums that showed the best results. For instance, figure **Figure 4.17.B** shows a screen donor that reacted poorly to CEF pool stimulation, and in which serums 2, 7 and 8 were the ones leading to a signal amplification above 50 SFCs, as the number of SFCs obtained were between 60 and 80. Similarly, in **Figure 4.17.C**, serums 2, 7 and 8 performed better, showing approximately 500 SFCs per million PBMCs. In addition, in figure **Figure 4.17.D**, serums 3, 4 and 8 were the ones that better amplified the response signal from this donor upon stimulation with CEF pool peptides. All the other serums tested on this donor were below the cut-off line, including the *in-house* used serum (serum 10).

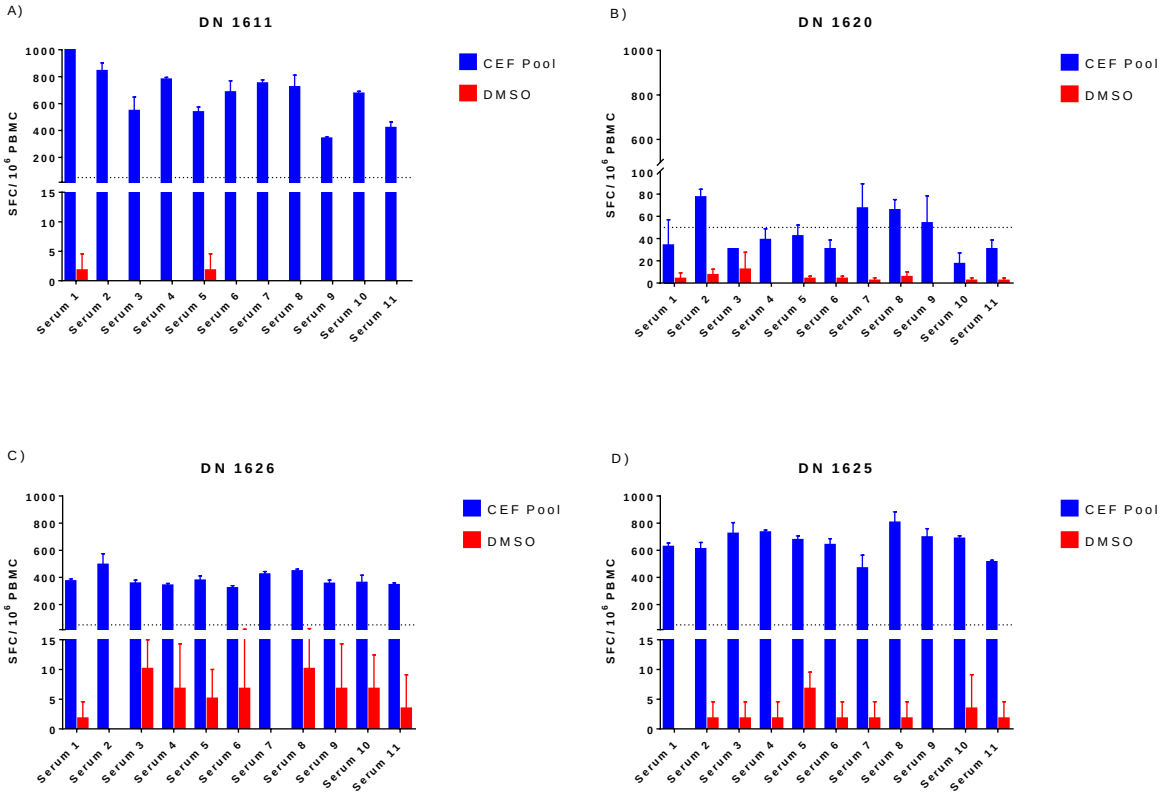

Figure 4.17. Quantification of spots forming cells in 1 million PBMCs in healthy donors **A)** DN 1611, **B)** DN 1620, **C)** DN 1626 and **D)** DN 1625 upon stimulation with a pool of CEF peptides.

Using the same assay, two screen donors were stimulated with individual CEF peptides, either peptides 19 and 32 (**Figure 4.18.A**) or peptides 6 and 7 **Figure 4.18.B**). These individual CEF peptides were chosen based on previous studies done with these donors and these CEF peptides as stimuli, showing signal amplification of the cellular markers tested here. Stimulation with CEF peptides 19 and 32 showed similar results among all the serums, even though the number of SFCs was slightly higher for CEF peptide 32 (approximately 400 SFCs per million PBMCs in all the serums) compared with CEF peptide 19 (200 SFCs per million PBMCs). Thus, results for this donor were not conclusive, and it is difficult to assess which serum was the best. With regards to donor 868, stimulation with CEF peptides 6 and 7 showed interesting results. Upon stimulation with CEF peptide 6, only serum 8 showed an increase in signal above the cut-off line. Nevertheless, upon stimulation with CEF peptide 7, almost all serums improved the signal, specially serums 1, 2, 7, 8, 9 and 10 which exhibited more than 1000 SFCs per million PBMCs.

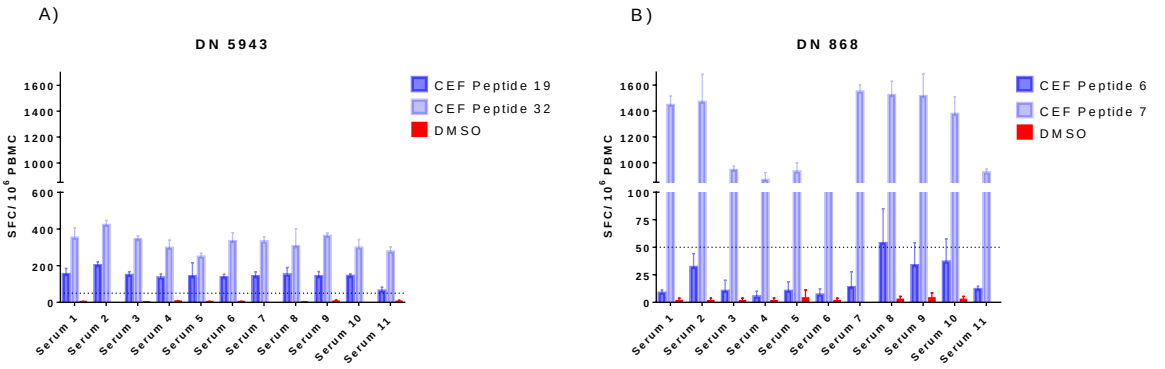

Figure 4.18. Quantification of spots forming cells in 1 million PBMCs in healthy donors **A)** DN 5943 upon stimulation with CEF peptides 19 and 32 and **B)** DN 868, upon stimulation with a CEF peptides 6 and 7.

4.5.2 Intracellular Staining (ICS)

Based on previous experiments performed in our lab group, we tested particular donors previously used in the assays above mentioned in combination with different serums, with the aim to evaluate their $CD8^+$ T cells expression levels of IFNγ, TNFα and IL-2. 10 serums were used in these assays for all donors. DMSO and CEF isotypes were integrated in the assay as background level controls. Also, donors were stimulated with either CEF pool peptides or individual CEF peptides, depending on the donor (**Figure 4.19**).

Screen donors DN 1611 and DN 1626 (**Figures 4.19.A and B**) demonstrated low background levels, meaning that without being stimulated, their $CD8^+$ T cells do not express IFNγ, or express it in a very low percentage. Even though the results from negative controls demonstrate that the assay worked, when stimulated with CEF pool peptides, these donors reacted in a positive manner, but still presented low levels of IFNγ expression by $CD8^+$ T cells. On screen donor DN 1611, serums 2, 4, 5, 9 and 10 seemed to slightly improve the signal and amplify the levels of expression of IFNγ by $CD8^+$ T cells, reaching almost 3% of cells expressing this marker. Despite showing lower levels of $IFN\gamma^+$ CD8 expression, screen donor DN 1626, also reacted upon stimulation. For this screen donor, serums 3, 4, 8 and 9 showed the best results, as with these serums around 0.5% of the CD8+ T cells expressed IFNγ. Screen donors DN 5943 and DN 868, which were stimulated with individual CEF peptides as previously described, showed different responses (**Figures 4.19.C and D)**. When stimulated with individual CEF peptides (19 and 32), screen donor DN 5943 reacted poorly and the expression levels of IFNγ by $CD8^+$ T cells were low. Nevertheless, CEF peptide 32 stimulation seemed to improve the results. However, if we compare the expression signal upon stimulation with this peptide to the high

background levels (DMSO condition), the expression of IFNγ becomes almost null. In this case serums 6 and 7 showed the best results. The values obtained for the stimulation of CEF peptide 19 were very similar amongst all the serums and comparable to the DMSO values (**Figure 4.19.C)**. Regarding donor DN 868, it was harder to evaluate IFNγ expression levels by $CD8^+$ T cells and distinguish which of the serums led to an amplification of IFNγ cellular expression. In this donor, stimulation with CEF peptide 6 resulted in similar expression levels between the different serums used, but once again these were comparable to the values obtained from the DMSO stimulation. However, when stimulated with CEF peptide 7, serums 2, 3, 8, 9 and 10 granted an amplification of the expression levels of IFNγ by $CD8^+$ T cells. In these conditions, between 1 and 2% of the analysed $CD8^+$ T cells expressed IFNγ. The exception was serum 10 (*in-house* serum), which obtained almost 6% of IFNγ expression by these cells. This difference might result from a variability in the donor response to the serums tested, or even from assay variability, which influences the results. Furthermore, this donor seemed to have no activation in the presence of serums 4 to 7 (**Figure 4.19.D)**.

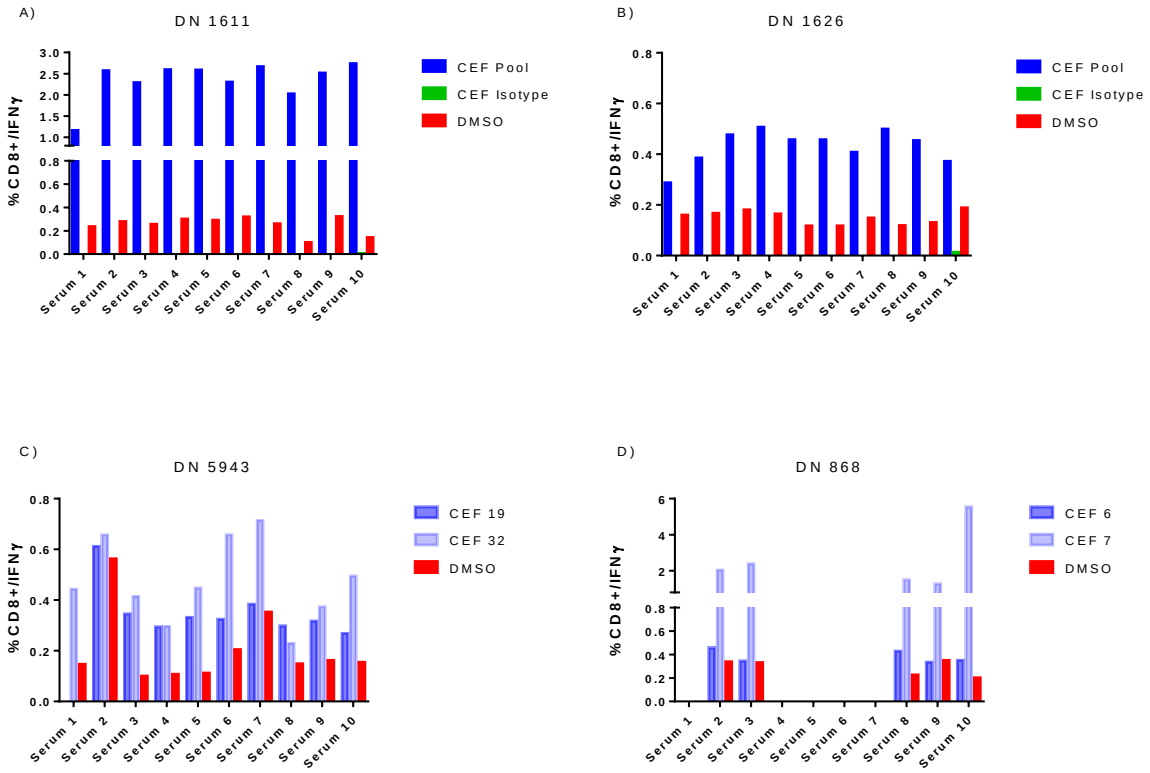

Figure 4.19. Quantification of IFNγ $^+$ $CD8^+$ T cells within the PBMC population (indicated in % from total PBMCs)) in healthy donors **A)** DN 1611 and **B)** DN1616 upon stimulation with a pool of CEF peptides, and in healthy donors **C)** DN 5943 and **D)** DN 868 upon stimulation with CEF peptides 6 and 7.

The expression of TNFα by $CD8^+$ T cells was also evaluated for the same screen donors, as shown in **Figure 4.20.** For this analysis, the same number of donors, controls and stimuli were used and compared among each other. It is necessary to state that the DMSO control showed higher background levels (overall between 0.2 and 0.8%) in comparison to the previous experiment, where IFNγ levels were analysed. Donor DN 1611 demonstrated activation after being stimulated with the entire pool of CEF peptides (**Figure 4.20.A**). Despite showing DMSO values around 0.5%, this donor reached values of $TNF\alpha^+$ $CD8^+$ T cells above 1.5%. Of note, serum 7 seemed to give the best results upon stimulation with the CEF pool (~3% of cells expressed the marker). On the other hand, screen donor DN 1626 did not respond well upon stimulation, showing low expression rates of TNFα, almost

equivalent to the DMSO control (**Figure 4.20.B**). Nonetheless, serum 8 reached TNFα expression percentage above 0.6%, demonstrating higher values than the other serums tested. This donor also demonstrated low TNFα expression levels upon stimulation with DMSO when serum 8 was used, suggesting that this serum presents the best results for this donor. As for screen donors DN 5943 and DN 868, the results varied mainly due to donor variability, stimulation with distinct individual CEF peptides and DMSO control values. As depicted in **Figure 4.20.C**, for donor DN 5943, only 3 serums (2, 7 and 10) demonstrated increase in the expression of TNFα upon stimulation. However, serum 2 and 3, presented results within the same range as the DMSO stimulation condition for both individual CEF peptides stimuli, meaning that no activation or increase in TNFα expression occurred in these two serums. On the other hand, although serum 10, did not seem to increase the levels of TNFα expression by $CD8^+$ T cells when stimulated with CEF peptide 19, upon stimulation with CEF peptide 32 the values of TNFα expression improved, and approximately 1.5% of the cells were shown to express it. Lastly, screen donor DN 868 responded well to the CEF peptide 7, but not to the CEF 6 peptide. Stimulation with CEF 6 peptide resulted in similar TNFα expression levels among the different serums (0.3% – 0.5% of $TNF\alpha^+$ cells), but these were comparable to the values obtained upon stimulation with the DMSO control. When stimulated with CEF peptide 7, serums 2, 3, 8, 9 and 10 responded well, and more than 1% of the $CD8^+$ T cells were also $TNF\alpha^+$. Like previously observed, no response was obtained with serums 1 and 4 to 7. Furthermore, the *in-house* serum (serum 10) was the serum candidate that showed better results upon stimulation for this screen donor (approximately 5% of the cells expressed TNFα upon stimulation with CEF peptide 7).

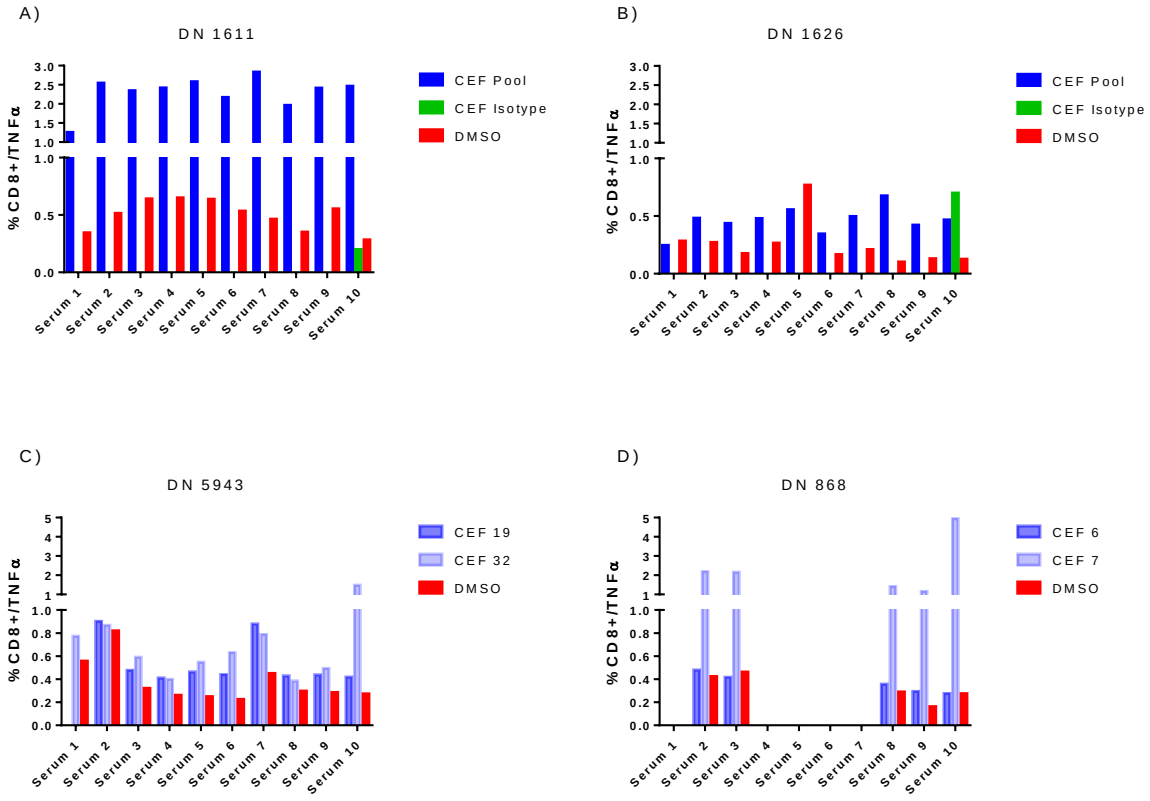

Figure 4.20. Quantification of $TNF\alpha^+$ $CD8^+$ T cells within the PBMC population (indicated in % from total PBMCs)) in healthy donors **A)** DN 1611 and **B)** DN1616 upon stimulation with a pool of CEF peptides, and in healthy donors **C)** DN 5943 and **D)** DN 868 upon stimulation with CEF peptides 6 and 7.

The last intracellular marker evaluated in this serum screen was IL-2, and once again by its expression was assessed in $CD8^+$ T cell within a PBMC population (**Figure 4.21**). All the stimuli, controls and number of serums tested here were the same used as the ones previously described for

the evaluation of IFNγ and TNFα expression patterns by $CD8^+$ T cells. Results indicate that the levels of IL-2 expression in this cell subset by are much lower when compared with IFNγ (**Figure 4.19**) and TNFα (**Figure 4.20**). However, it was still possible to distinguish IL-2 expression levels among the donors, and some serums potentiated an increase in IL-2 expression, when compared with the DMSO control values, which were very low in this experiment. For instance, all the serums seemed to amplify IL-2 expression in the same spectrum (0.2% to 0.5% for the cells expressed the marker) in screen donor DN 1611, upon stimulation with the CEF peptide pool. Although there was some variation between the responses to the different serums, this might be justified by a variation within the serums content. The same happened in screen donor DN 1626, where the results were similar among all the serums, even though IL-2 expression occurred at a lower range compared to donor DN 1611. Because of these overall lower levels, and due to the variation between serums, we concluded that practically all serums enhanced the signals obtained from this donor. As for screen donors DN 5943 and DN 868, which were stimulated with CEF individual peptides, their response to the presence of different serums was not distant from what we have seen for the other donors. Screen donor DN 5943 showed similar results for the two individual peptides among all the serums used (around 0.1% of $IL\text{-}2^+$ cells). On the other hand, screen donor DN 868 demonstrated a good response to CEF peptide 7 with serums 2, 3, 8 and 10. The latter is the *in-house* used serum, and showed the best results upon stimulation with this peptide, with around 0.5% of the cells expressing IL-2. Also, no response was obtained by serums 1 and 4 to 7, where no IL-2 expression was observed, and the same holds true upon stimulation with the CEF 6 peptide.

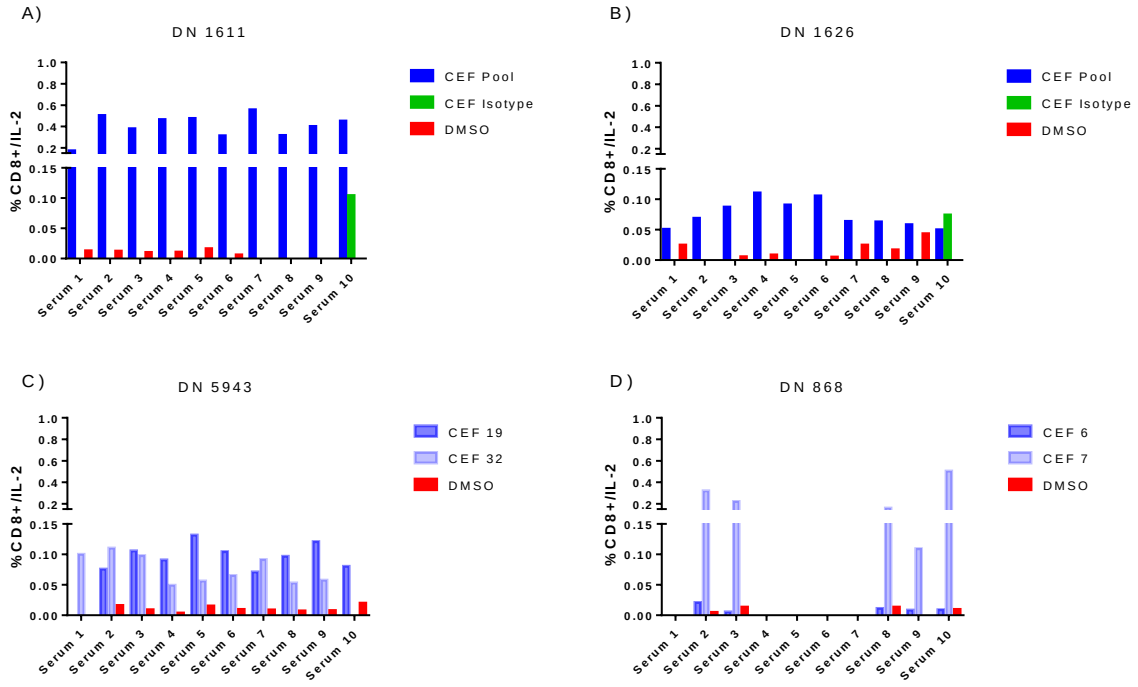

Figure 4.21. Quantification of $IL\text{-}2^+$ $CD8^+$ T cells within the PBMC population (indicated in % from total PBMCs) in healthy donors **A)** DN 1611 and **B)** DN1616 upon stimulation with a pool of CEF peptides, and in healthy donors **C)** DN 5943 and **D)** DN 868 upon stimulation with CEF peptides 6 and 7.

4.5.3 Proliferation Assay

In complementarity to the ELIspot assay, a proliferation assay was performed using some of the donors used in the previous experiments, and included 10 different serums previously tested. In this experimental procedure, all donors' PBMCs were stimulated with CEF pool peptides (**Figures 4.22**.**A** and **B**), and two of the donors were stimulated with individual CEF peptides (**Figures 4.22.C** and **D**). DMSO and Ki67 proliferation marker were also used, due to their well-stablished functionality as negative control and proliferation cell marker, respectively. FMOs for the Ki67 marker were also prepared for all donors as a negative control for the assay. In this assay, the percentage of $CD8^+$ T cells within PBMCs was evaluated based on the expression of the Ki67 cellular marker, i.e. based on these cells ability to proliferate after 5 to 7 days. The negative control, DMSO, showed the expected results for all the tested donors and serums (**Figures 4.22.A, B, C and D**). However, there were some exceptions: serum 10 on figure **Figure 4.22.A**, serum 9 on **Figures 4.22.C** and serum 4 on **Figures 4.22.D** responded in a relatively high manner, demonstrating high percentage of background in comparison to the other serums tested.

In **Figures 4.22.A** and **B**, there is a clear amplification of Ki67 signalling by $CD8^+$ T cells when stimulated. Serums 3, 4, 6, 9 and 10 (20 to 30% of cells express Ki67) for screen donor DN 1611 and serums 3, 4, 5, 6, 8 and 9 (20 to 40% of Ki67 expressing cells) for screen donor DN 1626 presented the best proliferation results for these donors. In particular, we noticed that serum 4 granted the best results among all tested serums for donor DN1626, reaching values of Ki67 expression by $CD8^+$ T cells of above 40%.

As for screen donors DN 5943 and DN 868, these were stimulated with individual CEF peptides, both 19 and 32 in the case of DN 5943, or 6 and 7 for DN 868. Screen donor DN 5943 proliferation levels seemed to be higher (10 to 20% of the cells expressed Ki67) compared to screen donor DN 868. In this donor, we observed that there was signal amplification in serums 3, 4, 5, 6 and 8, independently from the CEF individual peptide used as stimulus. Serum 10 was discarded for this donor's results, since both background levels and FMO percentage levels were too high. On the opposite, screen donor DN 868, showed very low levels of proliferation and this donor did not seem to be stimulated by any of the individual CEF peptides tested. It also did not present signalling amplification with any of the serums used. Only serum 3 and 6 show proliferative response for this donor upon stimulation with CEF peptide 7, where approximately 20 and 30% of the $CD8^+$ T cells expressing the Ki67 marker, respectively. However, when stimulated with CEF peptide 6, no response was obtained for any of the serums tested.

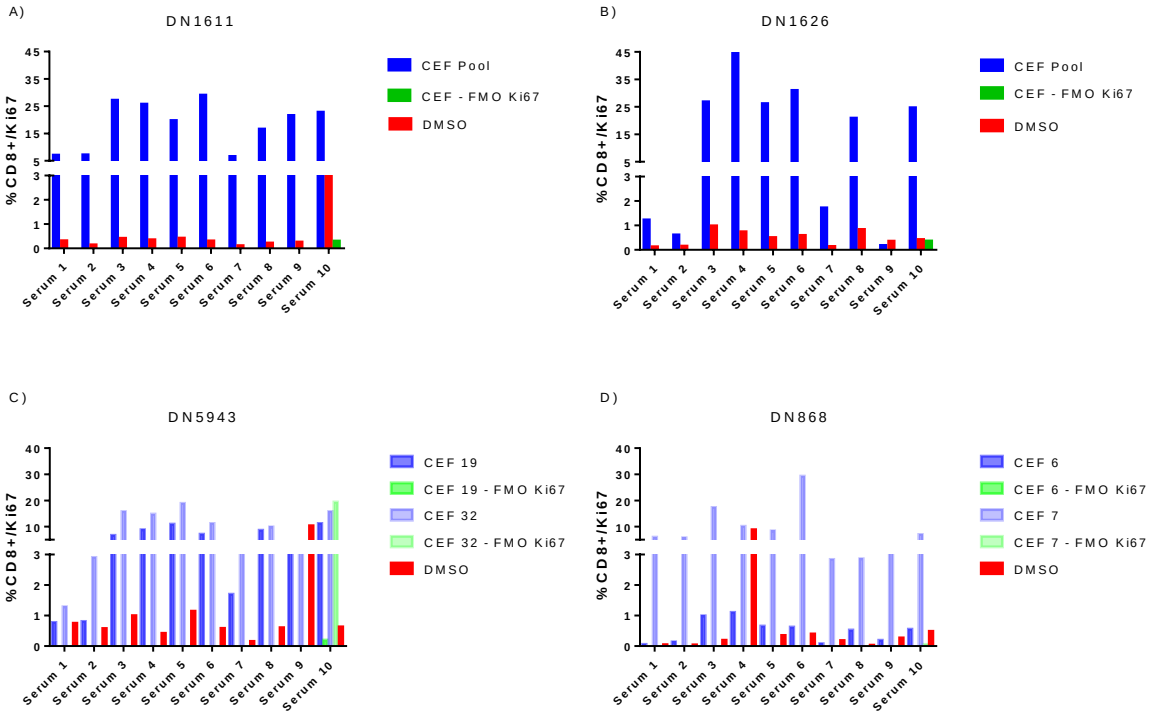

Figure 4.22. Quantification of $CD8^+$ T cells based on the proliferation marker Ki67 (indicated in % from total PBMCs) in healthy donors **A)** DN 1611 and **B)** DN1616 upon stimulation with a pool of CEF peptides, and in healthy donors C**)** DN 5943 and D) DN 868 upon stimulation with CEF peptides 6 and 7.

Chapter 5 – Discussion & Conclusions

The aim of this thesis project was to characterize immunologically MAIT cells in chronically infected HBV patients in comparison to MAIT cells from healthy donors. For that purpose, MAIT cells from healthy and chronic HBV patients were evaluated and compared for their phenotypic signature and their functionality, when stimulated with IL-12 and IL-18 recombinant cytokines. In this project, three main questions were addressed: **1)** Do MAIT cells show phenotypic differences in healthy and chronic HBV patients?, **2)** Are MAIT cells still functional in a chronic HBV infected patients? and **3)** Can MAIT cell be used as a new immunotherapeutic approach for HBV cure?

The experimental procedures and consequent results obtained in this project gave us a better understanding about MAIT cells and how to address the questions previously made, as well as to give input on the next experimental steps to perform using MAIT cells.

Gating Strategy, FMO's

A gating strategy was successfully set up for MAIT cells according to these cells specific markers, CD161 and TCRVα7.2. MAIT cells were gated on a lymphocyte population and used on subsequent assays (See **Materials and Methods** section). The gating strategy used within this project is in compliance to gating strategies used by other research groups[152]. At the same time, we successfully optimize the different antibody panels used in this project by performing FMO's experiments. This qualifies this gating strategy for every antibody used in the identification of our MAIT cells populations and subpopulations, as the FMO controls ensure any spread of the fluorochromes into the channels of interest, properly identified. The aim was to obtain accurate results and with the smallest deviation possible when characterizing MAIT cells phenotype and functional ability.

IL-12/IL-18 out titration experiments

It is known that MAIT cells are activated in a TCR dependent manner due to the presentation of pyrimidine intermediates such as 5-OE-RU or 5-OP-RU[105,106,149,211,212]. These can also be denominated as bacteria-born small compounds, derived from the riboflavin synthesis pathway, which is present in bacteria such as *Escherichia coli*[149]. However, MAIT cells can also be activated in a TCR-independent manner when stimulated with innate cytokines IL-12 and IL-18[213,214]. This occurs because MAIT cells express interleukin receptors correspondent to the cytokines IL-12R and IL-18R[107,123,160,200]. Some reports describe that is possible to activate MAIT cells through their stimulation with other innate cytokines, such as IL-15 and IL-7[200,215,216] which we did not have time to test in our study. In the scope of this project, MAIT cells were stimulated IL-12 and IL-18 recombinant cytokines (**See Materials and Methods**). Thus, it was necessary to perform out-titration experiments where these cytokines were used in different concentrations (See **Materials and Methods** and **Results**). We tested different dilutions of recombinant cytokines IL-12 and IL-18 stimuli, using healthy patients PBMCs. Out of the 6 dilutions for each stimulus, we found that the concentrations 0.25, 1 and 10ng/mL, and 1, 10 and 50ng/mL covered the optimal range for MAIT cell stimulation for IL-12 and IL-18, respectively. Also, the stimuli concentrations chosen allowed a better evaluation of MAIT cells activation range and functionality, based on the measurement of specific cellular markers expression in a dose-dependent stimulation manner.

Surface Markers

MAIT cells were evaluated for their total percentage in healthy and chronic HBV patients, based on two specific cellular markers for these cells, CD161 and TCRVα7.2[217,218] (See **Materials and Methods** and **Results**). MAIT cells were stimulated with IL-12 and IL-18 recombinant cytokines at different concentrations, as these cytokines activate MAIT cells[213,214]. Also, these cells

were tested for their phenotypic signature as an approach to understand if the frequency of $CD4^+$, $CD8^+$ and $CD56^+$ MAIT cells differs among the two groups tested - healthy and chronically infected HBV patients.

The results obtained in this project suggest that the frequency of total MAIT cells in healthy and chronic HBV patients is similar after stimulation with IL-12 and IL-18 (**Figure 4.1**). In our study We show that total population of MAIT cells were similar between healthy and chronically infected patients, but also that there is no difference in the percentage of MAIT cells responding to IL-12/18 stimuli. Therefore, we are confident that this study is comparing the same MAIT cell populations between the two patient groups. Also, we observed that after 24 hours stimulation with IL-12 and IL-18, the percentage of total MAIT cells did not increase within the two groups. One of the reasons why the total number of MAIT cells remains identical among healthy and chronic HBV patients might be that 24 hours stimulation might not be sufficient to induce MAIT cells replication cycle that can show differences between the two groups tested, since the conditions used were not suppressive. Nonetheless, HBV infection apparently does not promote MAIT cells death, which from a therapeutic development perspective is a positive remark for using these cells in the future as a therapy for HBV. Also, by limiting the stimulation time of MAIT cells (24 hours) we were able to restrict the number of variables used in this project and can directly compare healthy and chronic HBV patients. However, it is important to mention that the percentage of total MAIT cells in each donor individually presents variation, which suggests variability upon stimulation with IL-12 and Il-18 recombinant cytokines. This variable needs to be taken into account when working with human samples, in this case PBMCs. Some reports[174,175,219–221] indicate that the frequency of total MAIT cells ($CD3^+$ $CD161^+$ $TCRV\alpha7.2^+$) decreases during human viral infections such as human immunodeficiency virus (HIV) infections or co-infections like HIV/ *M. Tuberculosis*[221,222]. However, our results show the contrary in regards to hepatitis B patients and are supported by Boeijen and colleagues[223], where the same results were obtained with regards to the total MAIT cells percentage when these cells were sorted using specific cellular markers, CD161 and TCRVα7.2. At the same time, flow cytometry analysis of our samples confirmed that MAIT cells are a subset of innate-like T cells[214], showing a $CD3^+$ $CD161^+$ $TCRV\alpha7.2^+$ phenotype after IL-12 and IL-18 stimulation. Also, confirmed that the percentage of MAIT cells within the peripheral blood is comprised between 0 and 10%[115,212,224]. Despite these facts, we could argue that the variation in healthy and CHB MAIT cells baseline is high, but demonstrate an overlap when analysing their frequency. However, due to a low number of patients tested and consequent poor statistical data, it is not entirely correct to make this claim. For example, donor HBV1 shows less 20% $CD8^+$ and 4% $CD56^+$ MAIT cells expression percentages than healthy volunteers, and donor HBV3 presents the same pattern for $CD8^+$ MAIT cells expression. Therefore, further studies are needed to reproduce these results in bigger cohorts in the future, which would allow a statistical and comprehensive analysis of the levels of MAIT cells between healthy and HBV chronic donors.

We further investigated whether MAIT cells present a similar phenotypic signature as their parental population. As MAIT cells are known to be $CD3^+$ lymphocytes, we evaluated whether these cells could be CD4+ and CD8+, since this is the case with conventional T cell subpopulations. Importantly, while in the presence of a viral infection, as Hepatitis B infection, the existence of an active and sustained multi-epitope-specific $CD4^+$ and $CD8^+$ T cell response is necessary in order for the immune system to respond effectively[225]. However, in a situation of a chronic infection such as HBV, there is a failure in innate and adaptive response to clear the infection, which leads to an increased persistence by the virus within the host[225,226]. Subsequently, we tried to understand whether there are differences in the percentage of $CD4^+$/$CD8^+$ MAIT cells between healthy patients and chronically infected HBV patients (**Figure 4.2 and Figure 4.3**). These results are supported by Wakao and colleagues[212], as the authors state that in healthy humans MAIT cells are mainly $CD8^+$,

with a smaller percentage being $CD4^+$. Regarding chronic HBV patients, our results suggest the same, since exists a higher percentage of $CD8^+$ MAIT cells in comparison to $CD4^+$ MAIT cells, which strongly indicates that these cells could be used as a new therapeutic approach. $CD8^+$ T cells primary function is its cytotoxic function for clearing viral infection. MAIT cells in peripheral blood are mainly $CD8^+$ and that would mean that in chronic viral infections, like HBV specific T cell exhaustion, if we could transfect an HBV specific TCR to MAIT cells, possibly these could be used for HBV viral clearance and HBV specific hepatocytes killing.

The frequency of $CD4^+$ MAIT cells was measured and the results obtained indicate a slightly higher population in chronic HBV patients, when compared to health and after stimulation. Nonetheless, $CD4^+$ MAIT cells frequency is inversely related with their homologous population, $CD8^+$ MAIT cells. 2/3 of chronic HBV donors (HBV1 and HBV3), responded positively to both stimuli, reaching frequencies approximately two-fold higher than the frequencies we had observed in the healthy patients. Furthermore, while quantifying the frequency of $CD8^+$ MAIT cells from the both healthy and chronic HBV patients, it is possible to identify an increase in percentage, in both groups and in comparison to $CD4^+$ MAIT cells, previously discussed. Interestingly, chronic HBV patients (HBV1 and HBV3) show that 60% of the total MAIT cells tested in these donors are indeed $CD8^+$ MAIT cells and that there is a percentual decrease, when comparing to healthy patient's data. The sudden increase in $CD4^+$ MAIT cells frequency after stimulation and consequent decrease of $CD8^+$ MAIT cells frequency in chronically HBV infected patients, in contrast to healthy patients, could be directly related to the presence of a chronic viral infection[211]. In cases of HBV infection, $CD4^+$ T cells[227] support antiviral response signalling against the virus, whereas $CD8^+$ T cells role in focus on killing infected hepatocytes. Assuming a state of chronic HBV infection, in which the human immune system is continuously subjected to a state of latent infection $CD8^+$ T cells normally become exhausted, losing the capacity to act upon infected hepatocytes. MAIT cells, as one of T cells subset, show the same pattern in chronic HBV where $CD4^+$ and $CD8^+$ MAIT cells frequency is inversely related, curiously higher, and lower than healthy patients MAIT cells, respectively. Based on our results, and assuming that our observations regarding the total percentage of $CD3^+$ MAIT cells are correct, and that $CD4^+$ or $CD8^+$ MAIT cells derive from $CD3^+$ MAIT cells, is normal that if one subpopulation frequency shifts, the other automatically tends to compensate and change. In addition, the fact that patients are continuously infected may also influence the results, since the immune system might be exhausted. Importantly, when MAIT cells were stimulated with IL-12 and IL-18, $CD4^+$ MAIT cells frequency slightly increased, whereas $CD8^+$ MAIT cells frequencies remain identical, within each group.

MAIT cells were also evaluated for their frequency of CD56 expressing cells, as shown in **Figure 4.4**. This surface marker was tested in healthy and chronically HBV infected patients using the same experimental procedure as for the other surface markers, described before. Here, we tried to understand the extent of MAIT cells that are $CD56^+$, and to address if any differences appear when in a chronic viral infection environment. The results obtained suggest that $CD56^+$ MAIT cells are present both in healthy and chronic patients (between ≈10 and 35%, respectively). However, there was an exception, healthy donor HC 3, when stimulated with IL-18 at 50ng/mL showed almost 50% of $CD56^+$ MAIT cells. Still, no differences were observed when comparing the results for the two groups tested, even though all donors were prone to inherent variability, more patients' samples are needed to increase the statistical power of our results and possibly a net change in percentage that could normalize this variability and minimize its influence in our data. In resume, we concluded that MAIT cells phenotype, retrieved from human PMBCs, is also characterized by the surface expression of CD56, i.e., these cells are $CD56^+$.

Based on the results obtained from human PBMCs, we could conclude that MAIT cells display a phenotypic signature characterized by the presence of two surface markers, CD4 and CD8. Therefore, independently of their frequencies, which may vary from donor to donor, we concluded that MAIT cells contain separate $CD4^+$, $CD8^+$ and $CD56^+$ populations which were previously observed by Sandberg and colleagues[152].

***Ex Vivo* MAIT cell Activation**

Next, we evaluated MAIT cells for their expression of different molecules divided in three panels: 1) Checkpoint inhibitors, 2) Activation Markers and 3) Pro-Inflammatory Cytokines (See **Materials and Methods** and **Results**). Thus, in the scope of this project (See **Objectives**), we compared the activation state of MAIT cells retrieved from healthy patients and chronic patients infected with HBV. HBV chronic infection is characterized by a sustained level of T cell exhaustion[228]. For that purpose, MAIT cells were stimulated with IL-12 and IL-18 recombinant cytokines in three different concentrations each and evaluated for their expression levels of the following checkpoint inhibitors: PD-1, TIM3 and LAG3. There are several reports describing high levels of expression and the function of inhibitory receptors within an exhaustion environment in chronic viral infections[228–230]. In this project, we assessed MAIT cells for the extent of their exhaustion pattern and level. In **Figure 4.5**, the results obtained for MAIT cells expression of the checkpoint inhibitor marker PD-1 firstly show that MAIT cells retrieved from chronic HBV patients express higher PD-1 levels in comparison to MAIT cells isolated from healthy patients, with donor HBV2 reaching almost 80% of $PD\text{-}1^+$ MAIT cells. These results are in accordance to what is described in the literature, hence the percentage of $PD\text{-}1^+$ expressing MAIT cells in chronic HBV patients was expected to be higher than healthy controls due to the consistent viral infection and potentially higher MAIT cells exhaustion levels[231,232]. For instance, these results are supported by a study from Yong and colleagues[232], where the authors evaluated MAIT cells from chronic HBV patients with and without DNA viremia and compared them with MAIT cells from healthy patients. In this study, the authors show an increase in the levels of PD-1 expressing MAIT cells in chronic HBV patients in comparison to healthy controls[232]. Also, MAIT cells have been shown to be dysfunctional in chronic patients infected with the human immunodeficiency (HIV) virus [175,211] and co-infection of HIV and tuberculosis[221,224], correlated with PD-1 expression levels increment. Our results indicate that MAIT cells, when stimulated increase the rate of PD-1 expression and at the highest concentration of IL-12 (10ng/mL) the frequency of $PD\text{-}1^+$ MAIT cells reaches its highest value. In addition, these results suggest that, besides expressing PD-1, MAIT cells from chronic HBV patients are functional and may be resistant to exhaustion, based on $IFN\gamma^+$ MAIT cells results, discussed below. On one of the healthy patients, donor HC 1, this specific value was excluded from the results due to a low viability level and therefore failed our qualification criteria for using this sample (viability above 70%) (**Figure 4.5**). Based on our results MAIT cells from chronic HBV patients, these individuals present higher expression of PD-1 when stimulated, leading to an increase in these cells exhaustion state. However, upon the same stimulation parameters healthy patients MAIT cells also express PD-1, but at a lower degree of infection. This occurrence might happen, since PD-1 is also an activation marker, hence present at both test groups, with or without stimulation. Moreover, due to these cells' tissue localization on mucosal sites such as the liver and the amount and diversification of bacterial species within the same. Remarkably, PD-1 expression by MAIT cells in this organ is always present. Furthermore, all samples from both groups were compared to unstimulated samples (See **Materials and Methods**) which showed that the baseline level of PD-1 expression was comprised between 20% and 60%, meaning that without stimulation, MAIT cells from healthy and chronic HBV patients already express this checkpoint inhibitor.

Besides PD-1 checkpoint inhibitor, there are two other regulatory factors that are directly involved in immune dysfunction, TIM3 and LAG3. Therefore, the expression of these checkpoint inhibitors by MAIT cells was also studied[46,226]. TIM3 is known as a regulatory molecule expressed on T cells[226]. Upon chronic infection and T cells exhaustion, this inhibitory surface marker seems to be overexpressed and expressed at higher frequency[233–235]. Interestingly, some reports state that a blockade of TIM3 expression enhances cell proliferation and cytokine production[46,236]. Thus, this checkpoint inhibitor is seen as promising target for immunotherapy for infectious diseases and cancer[46,237].

In **Figure 4.6**, the results for MAIT cells expression of TIM3 checkpoint inhibitor are displayed. As shown for PD-1$^+$ MAIT cells expression, chronic HBV patients MAIT cells also demonstrate higher TIM3 expression percentages (30, 10 and 40% for donors HBV1, HBV2 and HBV3, respectively), in comparison to the baseline expression in healthy volunteers. However, the results for TIM3 are not as conclusive as for PD-1 expression, due to the fact that donor variability and variation upon IL-12 and IL-18 stimulation is higher than previously observed. When assessing the two groups tested, it is clear that only one of the healthy patients tested, donor HC2, demonstrated an increase in TIM3 expression by MAIT cells, reaching a percentage of 30% TIM3$^+$ MAIT cells upon stimulation with IL-12 at 10ng/mL. Donors HC1 and HC2 expression levels were not conclusive due to variation upon stimulation with IL-12 and IL-18 at different concentrations. On the other hand, chronic HBV donors HBV1 and HBV3 showed a positive response upon stimulation with these cytokines, where TIM3 expressing MAIT cells reached frequencies of 30 and 40%, respectively, and in comparison to healthy patients (below 5%) baseline expression. Donor HBV2 responded in a similar manner to the healthy patients HC1 and HC2. However, we could speculate that, upon stimulation with IL-12 recombinant cytokine, donor HBV2 responded positively, although at a lower extent compared to the other chronic HBV donors. In this donor, around 10% of the analysed MAIT cells expressed TIM3. Also, the results show that chronic HBV patient's response may vary depending on the stimuli concentration used, suggesting susceptibility upon stimulation. However, this variation might not be only associated to chronicity, since healthy volunteers demonstrate the same pattern, as well as strong individual variability.

Previously, Yuan Liu and colleagues indicated that CD4$^+$ and CD8$^+$ HBV-specific T cells showed an increase of TIM3 expression levels[46,238]. Despite having a low N value of donors in this project, our results suggest that the frequency of MAIT cells expressing TIM3 increases upon persistence of a viral infection (HBV). Overall, our data suggests that MAIT cells isolated from CHB patients PBMCs show higher expression frequencies of TIM3 in comparison to the healthy patients. The same appears to result in other chronic viral infection like HIV and HCV, where overexpression of TIM3 CD4$^+$ and CD8$^+$ T cells was associated to a progressive state of T cell dysfunction and consequent exhaustion [46,236,239–241]. These results are also in compliance to another research study from Yong and colleagues [232], where the authors showed an increase on TIM3$^+$ in Vα7.2 cells in chronic HBV patients in comparison to their healthy controls.

Lastly, the frequency of LAG3$^+$ expressing MAIT cells was evaluated on the same donors (See **Materials and Methods** and **Results**). LAG3 molecule is a member of the immunoglobulin family and carries a negative regulatory role in T cell exhaustion by suppressing T cells function[242]. Some reports give special attention to this checkpoint inhibitor function and involvement in T cells regulation[243–245]. Also, its role in effector T cells and Treg cells puts this marker in a position of potential target modulator for T cells regulatory mechanisms in autoimmunity, cancer and chronic infections such as HBV[246]. Our results regarding the comparative analysis of MAIT cells from healthy and chronic HBV patients, after stimulation with IL-12 and IL-18, shows discrepancies in the LAG3$^+$

frequencies among the two groups (**Figure 4.7**). Overall, chronic HBV patients, upon stimulation with cytokines IL-12 and IL-18 but depending on the stimulation concentration used, present 2 to 4 times higher $LAG3^+$ MAIT cell frequencies than healthy patients, whereas only healthy donor HC2 responds positively (2%) to one of the stimuli, IL-12 at 1ng/mL respectively. In 2/3 of the chronic HBV patients tested (HBV1 and HBV3), it seems that LAG3+ MAIT cells frequency progressively increases with IL-12 stimulation, whereas IL-18 recombinant cytokine only induces a positive stimulatory effect in chronic HBV donor HBV1. Importantly, chronic HBV donor HBV1 shows the highest $LAG3^+$ MAIT cells frequency (approximately 8%) in both stimuli, when compared to the other chronic HBV patients, as well as healthy patients. Interestingly, regarding the highest concentration used in both stimuli (10ng/mL for IL-12 and 50ng/mL for IL-18), these concentrations seem to be too high for this donor, and potentially no more MAIT cells are available to express LAG3, suggesting that such higher concentrations led MAIT cells to a state of apoptosis and could explain the low sample viability in this donor after stimulation. Ye and colleagues demonstrated that $CD8^+$ T cells in chronic HBV patients overexpress LAG3, in contrast to their healthy control values [247]. The same result was obtained in another report[243], where the authors concluded that chronic HIV patients $CD4^+$ and $CD8^+$ T cells LAG3 expression levels are upregulated, almost four times, in comparison to the healthy volunteer's samples. These two studies give sustain to our results, hence MAIT cells show the same response pattern as $CD4^+$ and $CD8^+$ T cells, since there is an overexpression of the checkpoint inhibitor molecule LAG3 in these cells. Also, our results suggest that upregulation of this molecule is mostly favoured when MAIT cells were stimulated with IL-12, both in healthy and chronic HBV patients. These findings could correlate to one report[242], in which the author describes the same, although this study was directed at conventional T cells from patients with follicular lymphoma, and states that there is a relation between the increase of LAG3 expression with the progression of the disease. We can conclude that, not only MAIT cells express LAG3 checkpoint inhibitor upon stimulation, but also that chronic HBV patients MAIT cells show upregulated $LAG3^+$ frequencies, when compared to the baseline expression from healthy patients. This could indicate that MAIT cells in chronic HBV patients are exhausted up to a certain extent, depending on the severity of the disease[228]. MAIT cells are known for expressing checkpoint inhibitory molecules, previously mentioned, in HBV and other chronic viral infection. This is strongly correlated with T cell exhaustion and suggests that MAIT cells in chronic HBV patients are exhausted or at least show this phenotypic state up to a certain point.

Activation Markers

Based on the fact that MAIT cells show expression of markers characteristic from exhaustion, we went further to understand if being exhausted meant no sign of being functional or whether these cells could still be activated when stimulated with IL-12 and IL-18 recombinant cytokines. For that purpose, we tested MAIT cells for different activation markers, **1)** early activation markers – CD69 and CD25 and **2)** late activation markers – CD38 and HLA-DR (**See Objective, Materials and Methods and Results Section**). It is imperative to mention, that the designations "early" and "late" are solely based on how long cells, in this case MAIT cells, were stimulated for and the time point at which these markers started to be expressed by MAIT cells. The baseline $CD25^+$ expression frequency on healthy patients MAIT cells is practically null in all donors, even upon stimulation with IL-12 and IL-18 recombinant cytokines (**Figure 4.8**). Nonetheless, healthy donor HC2 shows a positive response to both stimuli, displaying a maximum frequency of 10% of the total MAIT cells tested expressing CD25 activation marker. On the other hand, the data from chronic HBV patients suggests that MAIT cells are actively functional, since we observed an increase in CD25 expression. Donors HBV2 and HBV3 MAIT cells positively respond to IL-12 stimulation by expressing CD25, but at a low degree. As for IL-18 stimulation, it appears that donor HBV 3 was the only one showing

a positive activation, where $CD25^+$ expressing MAIT cells percentage progressively increases with the stimuli concentration and reaches 30% at 50ng/mL (**Figure 4.8**). Thus, when comparing healthy and chronic HBV patients $CD25^+$ expressing MAIT cells percentages, we can conclude that chronic HBV patients MAIT cells CD25 expression is upregulated, when compared to the baseline expression levels in healthy patients. Therefore, we can conclude that chronic HBV patients MAIT cells can be activated with IL-12 and IL-18 and demonstrate functionality based on CD25 expression. The previous statement is supported by Pushpa Hegde and colleagues, as the authors showed that peripheral blood MAIT cells from cirrhotic patients display an activated phenotype characterized by upregulation of CD25 expression marker, in comparison to the healthy controls [213]. The authors reported that the percentual difference between cirrhotic patients and controls of almost four times higher in cirrhotic patients, but contrary to our project MAIT cells were stimulated with PMA/ ionomycin[213]. Also, the authors assessed the CD25 baseline expression, as well as MAIT cells $CD25^+$ percentages after PMA/ionomycin stimulation. Our results suggest the same pattern, although it is necessary to increase the N value of the donors from each group to clarify our results, since despite the CD25 expression in MAIT cells from chronic patients is upregulated, when compared to healthy patients, the statistical difference between the two groups is tenuous. Nonetheless, several reports come to the same conclusion in other chronic viral infection such as hepatitis C (HCV) and HIV [102,175,218,248].

CD69 is a co-stimulatory receptor, as well as an early activation marker which function is correlated with the cytotoxic potential of lymphocytes, as well as cellular proliferation and cytokine production[225,249,250]. The role of this early activation marker in immune exhaustion is far less understood. However, since MAIT cells are found in systemic circulation and are enriched in the mucosa and liver, it is hypothesized that MAIT cells exhaustion could be in concordance to a reduction of CD69 in chronically infected HBV patients[225]. Also, this activation marker is known for being constitutively expressed by plentiful of cell types such as monocytes, NK cells, B cells and T cells[225,251,252]. In this project, we assessed whether the lack of effector cell function of exhausted MAIT cells could be a repercussion of reduced levels of CD69 in chronic HBV patients (See **Objective**). Our results show a clear downregulation of $CD69^+$ MAIT cell percentages in chronic HBV patients, compared to the baseline expression from healthy patients' MAIT cells (**Figure 4.10**). Healthy volunteers responded positively to both stimuli, and donor HC1 obtained the highest MAIT cell CD69 expression percentage, almost 80%, upon stimulation with IL-12 (at 10ng/mL) and IL-18 (10ng/mL). As for chronic HBV patients, the results were disparate and variable. Thus, only donor HBV1 should be assessed in more detail, since almost 60% of the total MAIT cells analysed expressed the early activation marker CD69. As for the other chronic HBV patients, donor HBV2 showed a similar response as healthy volunteers with no variation while stimulated with different stimuli concentrations and donor HBV3 only responded to IL-12 stimulation at 1ng/mL (**Figure 4.10**)**.** We could conclude that despite the downregulation in $CD69^+$ MAIT cells frequency in chronic HBV patients, MAIT cells are still functional and show activation as MAIT cells from healthy patients, even though at a lower degree**.** There are a few reports supporting our results and consequent analysis, though in our case the number of donors tested in both groups is low[173,175,225]. For instance, Yong and colleagues[225] assessed whether the decrease in number of cytokine-producing cells in chronic HBV patients, as well as cytokine levels produced in these patients, could be directly associated with the expression of CD69. The authors evaluated the expression level of this activation marker in MAIT cells through MFI and registered a reduction in CD69 expression levels by MAIT cells in chronic HBV patients in comparison to the healthy controls [225]. This remark correlates perfectly with the results obtained within this project, since the percentage of CD69 expressing MAIT cells decreased in HBV patients in comparison to healthy patients. However, some reports suggest

that, when in the presence of an exhausted environment associated with several infectious diseases, such as dengue virus, influenza virus, HCV and Systemic lupus erythematosus (SLE), this early activation marker is overexpressed by MAIT cells[109,211,253,254].

Above, we described and discussed the percentual expression levels of early activation markers – CD25 and CD69 – by MAIT cells in healthy and chronic HBV patients. Accordingly, we also compared the frequency of MAIT cells that express late activation markers – CD38 and HLA-DR – between both groups.

In this project we assessed MAIT cells CD38 expression and its impact in MAIT cells activation and function within a chronic infection such as HBV. The results obtained demonstrate that the baseline CD38 expression by MAIT cell from healthy patients is practically null, suggesting that this cellular marker is not accurate to conclude if MAIT cells are active or not in chronic HBV patients (**Figure 4.9**). However, healthy donor HC2 positively responded to IL-12 stimulation at 10ng/mL and approximately 20% of the total MAIT cells for this donor have shown CD38 expression**.** In contrast, chronic HBV patients MAIT cells CD38 expression is quite different, starting with the background levels, which are considered much higher in comparison to healthy patients and suggesting that a chronic HBV infection could influence CD38 expression and consequent MAIT cell activation and function. Furthermore, only donors HBV2 and HBV3 behaved in a dose-dependent manner in response to the increase in stimuli concentrations. Notably, chronic HBV donor HBV3 only showed this dose-response pattern upon stimulation with IL-18, where practically 40% of the total MAIT cells presented CD38 expression at the highest stimuli concentration. Chronic HBV donor HBV2 was responsive to both stimuli showing that almost 60% of the total MAIT cells tested express CD38. As for chronic HBV patient HBV1, its response to stimulation was null, since $CD38^+$ expressing MAIT cells frequency was equal to the background level (**Figure 4.9**). We can conclude that despite the low N value of donors tested, the late activation marker CD38 is expressed by MAIT cells from both healthy and chronic HBV patients. Also, we could advocate that MAIT cells from chronic HBV patients are active, functional and show overexpressed frequencies of CD38, which could be directly associated to a continuous viral infection, since this marker is considered a late activation marker. Boeijen and colleagues also claim in one of their reports that chronic HBV patients MAIT cells present higher CD38 expression levels, when compared to healthy controls[223]. Here, the authors also suggest a direct relation between $CD38^+$ MAIT cells increased frequencies to HBV disease progression, reaching it highest values in the inactive carrier phase. Another report evaluated the percentage of $CD38^+$ expressing MAIT cells in other viral infection such as Dengue and Influenza A virus[109]. In this case, the authors describe a slight upregulation in the expression of CD38 by MAIT cells in both diseases after 10 days of infection. Thus, these results might suggest that MAIT cells from chronic HBV patients might be able to be activated in a chronically viral infected environment.

Another late activation marker, HLA-DR, is constitutively expressed on the surface of B lymphocytes, monocytes and macrophages. This marker is well-described for being expressed at the late stages of activation on T and NK cells[255,256]. Also, the expression of HLA-DR on human T cells has been regarded primarily as a marker of activated T cells [257]. Following the previous analysis on MAIT cells activation pattern, the frequency of $HLA\text{-}DR^+$ MAIT cells was assessed. The objective was to get a better comprehension of this specific marker expression by MAIT cells and compare it to the previously tested activation markers in healthy and chronic HBV patients**.** The results obtained regarding the frequency of HLA-DR expressing MAIT cells in healthy and chronic HBV patients are similar (**Figure 4.11**). Nonetheless, there are results variations among donors from both groups, which can be directly related to the low number of donors and inter-donor variability, making it difficult to take solid conclusions. Based on HLA-DR+ expressing MAIT cells frequencies, our results

demonstrate that MAIT cells from healthy patients are functional and show activation when stimulated with both IL-12 and IL-18 recombinant cytokines. Also, in one of the healthy donors, HC2, we observed that more than 50% of the total MAIT cells tested expressed HLA-DR (**Figure 4.11**). It is true that the results for healthy and chronic HBV patients are similar for this marker, but it appears that chronic HBV patients MAIT cells show a slight upregulation of HLA-DR expression. For instance, chronic HBV donor HBV 2 responds in a dose-dependent manner to both stimuli as it reaches an HLA-DR$^+$ MAIT cell frequency of 80%, way above HLA-DR baseline expression found in healthy patients. The same pattern can be found in donor HBV3, whereas donor HBV1 response to stimulation is considered null and below the background level. Hengst and colleagues demonstrated in an increase in MAIT cells HLA-DR expression in hepatitis C infected patients, when compared to healthy controls[254]. Even though their experimental procedures were directed at understanding the influence of different therapies used in HCV patients and how they could affect different markers, their results are in alignment with this project. Other two reports, one in Human T-lymphotropic virus type 1 (HTLV-1) infection and another in HBV, both indicate an upregulation in HLA-DR expression by MAIT cells in infected patients, compared to health controls[232,258]. Further analysis should be made on MAIT cells HLA-DR expression, starting by increasing the stimulation time above 24 hours, since our results suggest the same as the previous studies mention, but are still not clear. Nonetheless, we can conclude that MAIT cells from chronic HBV patients do express the late activation marker HLA-DR, at a higher level than healthy patients MAIT cells, and are considered functional and active after being stimulated. Nevertheless, an upregulation in HLA-DR expression by MAIT cells in chronic infections could be an indicator of disease progression and state. As an extra remark, we were able to verify that in an exhausted environment, MAIT cells demonstrate an overexpression of several checkpoint inhibitory molecules, such as PD-1 and TIM3, which indicates that these cells present an exhausted phenotype. However, chronic HBV MAIT cells show activation and functionality in a chronic infection environment, as seen in this project by analysing different activation markers, upon stimulatory parameters. Nonetheless, not all activation markers evaluated on MAIT cells were expressed equally between both groups, and the fact that we observed a big inter-donor variation indicates the need to increase the number of samples analysed and repeat these experiments.

Pro-inflammatory cytokines

Under chronic viral infections or tumoral microenvironments, T cells activation mechanisms are downregulated as a feedback mechanism against the immune pathology, giving rise to a phenotype known as T cell exhaustion [259]. Processes such as proliferative capability, cytotoxic activity and pro-inflammatory cytokine production are normally decreased in functional exhausted T cells and are considered intrinsic characteristics of these cells phenotypic signature [259]. After showing that virally triggered MAIT cells are active, we went further with our analysis on MAIT cells to understand whether these cells can still show effector cell functions that may be significant to HBV chronic patients. MAIT cells were tested for different pro-inflammatory cytokines – IFNγ, TNFα and IL-2 – and their cytokine expression level was analysed and compared in healthy versus chronic HBV patients. IFNγ is the only type II interferon and it signals through the IFN-γ receptor (IFNGR). IFNγ production only takes place in specialized cells from the immune system, however their receptor (IFNGR) is present in most cells allowing this cytokine to bind all the major cell types and elicit a cellular response. Therefore, IFNγ is crucial in cellular immunity and establishes a bridge between innate and specific immune response pathways [260].We looked at pro-inflammatory cytokines in order to understand if MAIT cells are functional despite PD-1 and other checkpoint inhibitors upregulated levels. In addition, this project explored the concept that MAIT cells retain T cells functionality in a tolerizing environment. Our results show that there is no upregulation of IFNγ expressing MAIT cells in chronic HBV patients in comparison to the healthy volunteers, which is concurrent with the

statement that MAIT cells have no TCR receptors and therefore cannot recognize HBV infected hepatocytes (**Figure 4.12**). MAIT cells from healthy patients seem to respond positively towards IL-12 and IL-18 stimulation, but in a weak manner and showing low of IFNγ$^+$ MAIT cells percentages. Only donor HC2 shows a dose-response pattern when stimulated with IL-12 reaching the highest percentage (15%) of IFNγ$^+$ MAIT cell at 10ng/mL of stimulus. Likewise, only one chronic HBV donor (HBV2) shows a dose-response curve when stimulated with IL-12, reaching the same percentages of IFNγ$^+$ expressing MAIT cells seen in healthy donor HC2. However, donors HBV1 and HBV3 MAIT cells demonstrate a slight increase in IFNγ expression when stimulated with 10ng/mL and 1ng/mL of IL-12, upon which around 5% of the total MAIT cells analysed express IFNγ. Nonetheless, these donors MAIT cells were also subjected to IL-18 stimulation, but overall responded insufficiently. Despite the low N value and high variability, our results suggest that IL-12 stimulation forces MAIT cells to secrete more IFNγ in donors from both groups, but in similar levels when compared against each other. Several studies have focused on quantifying the levels IFNγ expression on MAIT cells retrieved from patients with chronic viral infection such as HBV, HCV, HIV or other chronic liver pathologies that lead to a cirrhosis or hepatocellular carcinoma state[213,218,232,248]. A recent paper concerning MAIT cell exhaustion in HBV infection shows that IFNγ expression is upregulated in MAIT cells from healthy patients, compared to a poor expression level in chronic HBV patients[232]. The authors' hypothesis is based on a relationship between the number of IFNγ$^+$ MAIT cells and the disease state and progression. Also, Xiao and colleagues proved MAIT cells involvement in viral infections clearance and showed that in chronic HCV patients these cells are activated in a cytokine-mediated manner, leading to an IFNγ upregulation[214]. In contrast to these and other reports [109,248,261], our results indicate that MAIT cells from chronic HBV patients show a similar degree of IFNγ expression as MAIT cells from healthy volunteers (**Figure 4.12**). In concordance to our results, Hegde and colleagues, while measuring the percentage of IFNγ$^+$ MAIT cells in healthy volunteer and cirrhotic patients, have found no significant differences in frequencies and activation response between the two groups[213]. MAIT cells show indeed effector cell function and production of IFNγ and there are references showing residual MAIT cells to be functionally active in HIV-infected patients, possibly contributing in mucosal inflammation by the release of pro-inflammatory cytokine such as IFNγ[175,224,262]. We can conclude that MAIT cells from chronic HBV patients have a similar activation and effector cell function as healthy MAIT cells. However, it is possible that IL-12 and IL-18 stimuli concentrations were not as optimal as we thought, since these recombinant cytokines are not only stimulators of MAIT cells but also influence IFNγ production by T and MAIT cells[109,218,263]. MAIT cells, contrary to normal CD8$^+$ T cells, show IFNγ and TNFα functions, even though being PD-1 positive, suggesting that MAIT cells are able to overcome exhaustion at a certain degree.

Besides expressing IFNγ, MAIT cells also show evidence of secreting TNFα in several diseases, such as alcoholic liver disease (ALD), Scrub Typhus, HCV and HBV[211,232,264–266]. TNFα is known for being a pleiotropic cytokine involved in a range of physiological mechanisms that regulate inflammation, anti-tumoral responses and homeostatic control of the immune system[267–269]. This pro-inflammatory cytokine is well-characterized for its protective endeavour against pathogens and as an effector cell product of CD4$^+$ and CD8$^+$ T cells or innate-like T cells such as MAIT cells, leading to the killing of infected cells[267]. The majority of the actions taken place by TNFα are mediated by the TNF receptor 1 (TNFR1), ubiquitously expressed by a large extent of cell types[267,270–272]. However, it is still unclear the role and effects of TNFα on T cells, though it is thought that this cytokine receptor is induced on T cells after activation[267]. Nevertheless, the expression levels of this cytokine in chronic HBV patients and healthy volunteers may be suggestive of a direct relation between MAIT cells exhaustion, activation and effector cell function upon infection. Therefore, in this project we aimed to understand the dynamics of TNFα pro-inflammatory cytokine expression in chronic HBV patients

(See Objective, Materials and Methods and Results Section). The results obtained regarding MAIT cells TNFα expression are considered similar for both groups (**Figure 4.13**). Only one healthy donor, HC1, positively responded to stimulation (IL-12) and shows an increment in TNF+ expressing MAIT cells frequency up to a maximum of 1% of the total MAIT cells evaluated. As for chronic HBV patients, MAIT cells show a slight TNFα expression increase, which also depends on inter-donor variability and stimulation concentration variations. Chronic HBV donors HBV2 and HBV3 act in a similar manner as healthy donors upon stimulation with IL-12, where it is observed that at the highest concentration MAIT cells express more TNFα. Furthermore, chronic donor HBV1 MAIT cells show approximately 3.5% TNFα expression, which is more than two times higher than the baseline expression in healthy patients and could be indicative of an increment in effector cell function by MAIT cells. Nonetheless, it is of the utmost importance to mention that the background expression levels for this donor were extremely high (almost 2%) and it should be noted that the number of $TNF\alpha^+$ MAIT cells was very low in both groups. Concerning IL-18 stimulation, no upregulated response in $TNF\alpha^+$ expression by MAIT cells was seen in both groups. There are several reports investigating MAIT cells' TNFα expression in patients suffering from multiple infectious diseases [218,232]. Yong and colleagues performed a functional assay assessing the potential impairment of MAIT cells in IFNγ and TNFα production on chronic HBV patients[232]. The authors observed a decreased $TNF\alpha^+$ MAIT cells frequency in chronic HBV patients, which is suggestive to a direct connection to the high expression level of PD-1 in infected patients MAIT cells, leading to the functionally impairment of MAIT cells [232]. Furthermore, in a report in HCV and antiviral therapy effect, it is shown a similar $TNF\alpha^+$ MAIT cells expression in HCV patients and healthy controls [218]. These findings are supported by a study performed by Laidlaw and colleagues showing that TNFα secretion is another effector function of MAIT cells, depite the fact that the experimental feature is slightly different, since cells were not stimulated with IL-12 or IL-18 but different TNFα concentrations were measured[266]. Interestingly, in HCV this pro-inflammatory cytokine can have an antiviral role in inhibiting the spread of the virus[211,266]. In a study about Scrub Typhus disease the authors concluded that MAIT cells deficient TNFα expression *in vitro* reflects disease severity and demonstrated an increase in TNFα expression due to a consequent disease remission towards a healthy state[265]. Also, $TNF\alpha^+$ MAIT cells production impairment is related to elevated CD69 expression in this specific disease[265]. As a remark, our results show a downregulation of $CD69^+$ MAIT cells percentages in chronic HBV patients, as well as poor production of TNFα. Nevertheless, chronic HBV patients are constantly on antivirals which may lead to a change in MAIT cells phenotype, as seen in cirrhotic patients subjected to long-term prophylactic antibiotic therapy[213]. In conclusion, our results are not enough to understand the degree of potential changes in TNFα expression by MAIT cells on chronic HBV patients, and to comprehend whether it may help clear the virus during a possible disease remission. However, our project is solely a piece of the puzzle, and these results should be optimized in the future by increasing the number of donors tested and by stimulating MAIT cells with IL-12 and IL-18 together. Despite this, our findings are still supportive of MAIT cells having a role in immune mechanisms and pro-inflammatory properties.

MAIT cells exert its role in protective immunity against microbial infections through the production of effector molecules, such as IFNγ, TNFα and IL-2, and others like IL-17 and GrzB, which were not tested within this project[255,273]. We measured $IL\text{-}2^+$ MAIT cells frequency in healthy and chronic HBV patients (**Figure 4.14**), since IL-2 is a pleiotropic cytokine, primarily produced by $CD4^+$ T cells, although there is evidence of being secreted by activated dendritic cells (DCs), $CD8^+$ T cells and NKT cells, though at lower levels[273,274]. Our results show limited IL-2 production by healthy and chronic HBV patients MAIT cells upon IL-12 and IL-18 stimulation, which might be influenced by inherent inter-donor variability in both groups. Upon IL-12 stimulation, the frequency of $IL\text{-}2^+$ MAIT cells in healthy and CHB patients is considered disparate, since healthy patients do not

demonstrate any positive response or increase in frequency to situation, whereas chronic HBV patients, namely donors HBV 1 and HBV3 show an increase in IL-2^+ MAIT cells percentage. Nonetheless, due to a high background level, chronic donor HBV1 response can have been misdirected and in contrast, this donor lacks a positive response to the stimulus. On the other hand, when evaluating MAIT cells from healthy and chronic HBV patients to IL-18 stimulation, the results are slightly different, since in both groups there are donors which respond positively and show an increase in IL-2^+ MAIT cells frequency. In this case, two out of three healthy donors show a positive response to IL-18 stimulation and two out of three chronic HBV patients show the same response (**Figure 4.14**). Interestingly, healthy donor HC3 at the highest IL-18 concentration shows 1% of the total IL-2^+ MAIT cells, which is way above the results obtained for all the chronic HBV patients. There is not much of a difference in our results, when comparing both groups, it is possible to see their response for the three IL-18 concentrations used is practically the same. As $IL2^+$ MAIT cells analysis in this project was subjected to several variables, low number of donors, inter-donor variability and low cytokine expression, it is preposterous to assume differences between healthy and chronic HBV patients in IL2 expression. Thus, based on this cytokine expression alone, it is not possible to infer whether chronic HBV patients MAIT cells still demonstrate effector cell functions within this exhausted environment. Besides the data that we generated, there are a few reports debating IL-2 presence and expression by MAIT cells, as well as its possible role as novel therapeutic target for different diseases[280]. For instance, in 2014, Serriari and colleagues reported that in inflammatory bowel diseases like Crohn's disease (CD) and Ulcerative colitis (UC), characterized by an immune response deregulation targeting the gut bacterial flora, MAIT cells activation during pathology could translate in a functional response[281]. The authors also claim that MAIT cells produce predominantly IFNγ, TNFα and IL-2, when stimulated *in vitro*, but when activated they might express more pro-inflammatory cytokines or change the set of effector molecules secreted[281]. In their report, it is also shown that $IL2^+$ expressing MAIT cells frequency is lower in healthy patients, when compared to infected ones.

In contrast, our results suggest similar but low $IL2^+$ MAIT cells frequencies in both groups, which might make sense since IL2 expression is normally showed in low basal levels in resting T cells[275]. However, there is evidence showing that upon stimulation, T cells show an increase of IL-2 expression, which might be correlated to CD25 activation marker expression[255,282]. Curiously, CD25 is the alpha chain of the trimeric IL-2 receptor and it is expressed constitutively in the surface of several peripheral blood lymphocytes such as regulatory or memory T cells, making this relationship feasible[255]. In this project, we found that MAIT cells do express CD25, known for its role as an activation marker, and that chronic HBV patients show a slight upregulation of $CD25^+$ expressing MAIT cells. Interestingly, we also found that chronic HBV patients MAIT cells seem to be more activated, and as a consequence show higher IL-2 production, although not confirmed for all donors. As a concluding remark, even though we observed that MAIT cells from chronic HBV patients overexpress exhaustion markers such as PD-1 and TIM3, these cells are still active, as confirmed in experiments where different activation markers were tested. As a consequently of this activation, MAIT cells are able to increase their frequencies of effector cell molecules like IFNγ, TNFα and last IL-2, although the outcome was dependent on the evaluated cytokine.

Functional Assay – C1R and Isolated MAIT cells

To evaluate whether MAIT cells are still functional or show an immune suppression similar to T cells in chronic HBV patients, we used two different procedures to isolate MAIT cells from human PBMCs (**Figure 4.15** and **Figure 4.16**). The objective was to couple isolated MAIT cells together with a B lymphoblast cell line - HMy2.CIR [C1R, HMy2.C1R] –, which functions as an antigen presenting cell (APC) line, and surveys if this interaction results in an upregulation of MAIT cells function or in modifications in these cells' activation pattern. Nonetheless, we unsuccessfully isolated MAIT cells from human PBMCs when using a magnetic beads isolation kit (**Figure 4.15**). As previously mentioned, MAIT cells can be identified and isolated with the help of two different

markers: CD161 and TCRVα7.2. However, and probably due to a lack of optimization of the protocol, we could not isolate MAIT cells, hence double positive MAIT cells for these markers were lost along the process and retained in the negative fraction of the columns, leading to a inexistent or decreased percentage of isolated MAIT cells in the end. Even when using a death cell removal kit upfront to try and compensate the amount of negative MAIT cells in the positive isolated fraction the result was the same as before. The major concern using this isolation technique was indeed the low cell viability associated to loss of cells during the entire experimental procedure and the presence of negative MAIT cells within the positive MAIT cells fraction. Therefore, we then used a cell sorter to separate MAIT cells from human PBMCs. Figure 4.16 shows a successful isolation of MAIT cells population (double positive for CD161 and TCRVα7.2) from human PBMCs with a purity of 99.3% after sorting.

Once we isolated a sufficient amount of MAIT cells, these cells were put together with the HMy2.CIR [C1R, HMy2.C1R] cell line and tested for different cytokines and chemokines using a Luminex Kit (See **Matherials and Methods** and **Results**). This cell line is well-descrided as an antigen presenting cell (APC) line and able to promote the delivery of an MR1 agonistic compound (Diclofenac) to MAIT cells, known for activating these cells. However, the assay did not show the expected results since the expression levels of all the cytokines and chemokines tested with Luminex were almost inhexistent or null. The same observations were made when testing different ratios between the C1R cell line and sorted MAIT cells. Still, our results suggest that sorted MAIT cells together with the C1R cell line already show some degree of activation. Nevertheless, the presence of diclofenac does not show any enhancement of this activation state, and it is possible that the compound concentrations or the ratios applied were not optimal to allow an upregulation of MAIT cells' activation.

Serum Screening

As a secondary objective within this project, we performed a serum screen for PBMCs culture media, where eleven different commercially available serums were tested in three different assays – Elispot, Proliferation and ICS – using previously described methods (See Materials and Methods Section). Since this project was mainly created to evaluate and compare healthy patients and chronically HBV patients, we first used the Elispot assay, due to its high sensitivity (**Figure 4.17** and **Figure 4.18**). Here, we assessed the response of six healthy donors upon stimulation CEF pool and individual peptides, translated by the number of spot forming cells. As mentioned in the results section, the healthy patients used were classified as low and intermediate responders. Our aim was to find at least one serum that could amplify our donors signal, i.e., increase the number of spot forming cells in comparison to the negative controls used. The purpose of using solely low and intermediate responders was due to fact that chronic HBV patients are low responders, demonstrating very low to inhexistent response in assays such as Elispot. The results obtained were broad, suggesting that more than one serum could provide the maximum amplification signal. Nonetheless, serums 2, 7 and 8 showed the best resuts, having in thought the controls and stimulation parameters used, as well as an increase in the number of spot forming cells in each donor tested.

Additionally, we performed two different assays: ICS and proliferation assay (**Figures 4.19-4.22**). Respectively, $CD8^+$ T cells gated out of the human PBMCs donors were tested for their pro-inflammatory cytokines – IFNγ, TNFα and IL-2 – expression rate, as well as spread ability regarding the Ki67 proliferation marker, while maintaining the same stimulus conditions as before. As T cell are known to express pro-inflammatory cytokines when in an exhaustion environment such as HBV, we sought to investigate which serum out of the eleven tested indicated the highest expression rate on $CD8^+$ T cells. It matters to mention that the donors tested have shown high inter-donor variability upon stimulation, as well as a high variability in cytokine expression, rendering difficult to take solid conclusions about which serum would be the best for further studies. Nevertheless, among the

different serums subjected to analysis, serums 2, 3 and 8 increase the overall expression of the different cytokines tested on $CD8^+$ T cells. As for the proliferation ability of $CD8^+$ T cells, the results were highly variable among the screen donors tested. Their results were once again disparate, since each donor, showed different Ki67 proliferation degrees upon stimulation, which is likely associated with the inherent inter-donor variability, as previously mentioned. However, $CD8^+$ T cells seemed to increase their proliferation performance when in the presence of serums 4, 6 and 8. As a concluding remark, after analysing all the data from the different assays performed, serum 8 was chosen since provided the finest and more optimal results. Also, this serum would be used afterwards in different immune assays within the research group.

Conclusions

Although the work developed throughout this thesis provided an initial characterization of MAIT cells in the context of chronic HBV, further investigation is essential to better understand the role and functionality of these cells within a chronic infection environment, as well as their potential as a new immunotherapeutic approach.

For instance, a similar preliminary characterization as the one described in this thesis should be done using liver samples from both control and chronic HBV patients. Such experiment would provide an important comparative analysis between peripheral blood MAIT cells data and those present in the liver, since it is known that MAIT cells comprise approximately 40% of the total number of T cells in this organ. Additionally, this would be crucial to confirm the hypothesis that MAIT cells can overcome T cell exhaustion and remain functional in chronic HBV patients.

After establishing the dynamics and function of MAIT cells during a chronic infection, there is also a need to confirm whether these cells can be used as immunotherapy against chronic HBV. Since MAIT cells are naturally unable to recognize and kill HBV infected hepatocytes due to the lack of an HBV specific TCR, initial experiments might focus on the coupling of an HBV specific TCR to functional MAIT cells and delivery of these constructs into the affected organs/ cells.

Although some studies with patient and *in vitro* experiments have shown that MAIT cells might sense a viral infection through a cytokine-driven network, there is still a lot of basic research needed to validate these cells as a therapeutic approach for chronic HBV and other viral diseases. For instance, it is not clear yet whether MAIT cell activation driven by a viral infection is protective or deleterious for the host, and thus new models are needed to better modulate the activity of these cells and its consequences. It is also important to confirm such data in *in vivo* models. Furthermore, it is essential to prove the specificity of MAIT cells responses. Since these cells can be activated merely by cytokines, in the absence of an APC, it is theoretically possible that these cells respond to stimuli without any local infection. Thus, experiments showing that activation of these cells in a TCR-independent manner could lead to the death of virally infected cells would be very interesting. Finally, we still need to understand how the activation of MAIT cells might lead to antiviral effects and define how much MAIT cells participate in early events of adaptive and other innate immune responses. Again, in vivo models would be better suited to address this issue.

In conclusion, we show that MAIT cells in chronically infected HBV patients are activated by IL-12 and IL-18 stimulation as healthy controls, they produce cytokines necessary for cytotoxic activity at comparable levels as healthy controls despite having upregulated exhaustion markers PD-1, TIM-3 and LAG3. The canonical description of exhausted T cells involves the loss of IFNγ and TNFα production with upregulated exhaustion markers. This study shows that MAIT cells do not follow this classical exhaustion pathway and retain functionality in tolerizing environments, meaning MAIT cells are a promising target for immune-cell based therapies in chronic infections or tumour treatment where the active site of disease is a tolerizing environment. Also, since we used 3 separate flow panels, to test MAIT cells regarding their activation, exhaustion and cytokine expression is important

to state that even though these panels were used individually, all of them evaluated stimulated MAIT cells. Moreover, the final goal of this project was to assess if MAIT cells from chronically infected HBV patients were as functional as MAIT cells from healthy patients, not better or in high percentage.

It is clear that a lot of questions are still unanswered regarding the role of MAIT cells, but since these cells represent a substantial fraction of circulating and tissue specific T cells in the human body, further investigation is needed to settle what their contribution is in the immune response against HBV and other viral infections.

Chapter 6 – References

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