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# Functional and phenotypic characterization of CD4 T cells involved in immunopathogenesis of hepatitis B virus infection

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# Zusammenfassung

Das humane Hepatitis B Virus (HBV) ist ein nicht-zytopathisches, behülltes DNA-Virus, das einen starken Lebertropismus aufweist. Die Infektion mit HBV stellt ein globales Gesundheitsproblem dar, da jährlich mehr als 780.000 Menschen an ihren Folgen sterben und derzeit über 240 Millionen Menschen chronisch infiziert sind. Die chronische Infektion mit HBV ist mit einer progressiven Entzündung und Schädigung der Leber (Hepatitis B) assoziiert und kann dadurch zu einer Leberzirrhose oder einem hepatozellulären Karzinom führen. Die gegenwärtigen Standardtherapien zur Behandlung der chronischen Hepatitis B führen nur in seltenen Fällen zur Ausheilung. Aus diesem Grund werden dringend neue Therapieansätze zur Behandlung dieser chronischen Erkrankung benötigt. Da eine chronische Hepatitis B mit unzureichenden HBV-spezifischen T-Zellantworten verbunden ist, stellen immuntherapeutische Strategien zur Induktion von effizienten antiviralen T-Zellantworten einen vielversprechenden Ansatzpunkt dar.

Zur Entwicklung neuer Immuntherapien ist die Identifikation von effektiven HBV-spezifischen T-Zellantworten maßgeblich. Eine wichtige Strategie zur Identifikation von effektiven HBV-spezifischen T-Zellantworten stellt das Monitoring von T-Zellen bei Patienten mit selbst-limitierender und chronischer Hepatitis B dar. Des Weiteren wird das Monitoring von T-Zellen in klinischen Studien verwendet, um die Wirksamkeit von T-Zell induzierenden Therapien bei Patienten mit chronischer Hepatitis B nachzuweisen. Daher beschäftigte sich die vorliegende Arbeit mit der Etablierung einer sensitiven, präzisen und verlässlichen Methode zur Analyse von HBV-spezifischen T-Zellen. Die *in vitro* Expansion von mononukleären Zellen des peripheren Blutes (PBMC) ist eine weit verbreitete Methode, um die geringe Anzahl HBV-spezifischer T-Zellen und damit die Test-Sensitivität zu erhöhen. Um den Effekt einer 10-tägigen *in vitro* Expansion auf HBV-spezifische T-Zellen zu untersuchen, wurde im Rahmen dieser Arbeit die Frequenz und Funktionalität von HBV Surface- (HBs) und Core- (HBc) spezifischen T-Zellen *ex vivo* und nach Expansion bei 41 Spendern mit unterschiedlichem HBV Infektionsstatus (akut, chronisch und ausgeheilt) verglichen. Die Reaktivität der T-Zellen wurde mittels intrazellulärer Zytokinfärbung (IL-2, IFN-γ, TNF-α) ermittelt. Die Expansion führte zu einer signifikanten Erhöhung der Anzahl HBc-spezifischer CD4/CD8 T-Zellen, HBs-spezifischer CD4 T-Zellen, aber nicht HBs-spezifischer CD8 T-Zellen. Die Kapazität HBV-spezifischer T-Zellen *in vitro* zu proliferieren korrelierte dabei nur schwach mit der detektierten *ex vivo* Frequenz und war unabhängig vom HBV Infektionsstatus des Spenders. Auch in der Funktionalität (Zytokinprofil) HBV-spezifischer T-Zellen wurden Unterschiede *ex vivo* und nach Expansion festgestellt. Die proliferative Kapazität *in vitro* korrelierte dabei mit dem Grad der Veränderung des Zytokinprofils. Insgesamt resultierte die

Expansion nicht in einer Steigerung der Test-Sensitivität. Basierend auf diesen Daten lässt sich schlussfolgern, dass die *ex vivo* Analyse von HBV-spezifischen T-Zellen möglicherweise die *in vivo* T-Zell-Reaktivität am besten beurteilt, was in zukünftigen Studien berücksichtigt werden sollte.

Des Weiteren wurde in dieser Arbeit eine neue CD4 T-Zellpopulation bei Patienten mit chronischer Hepatitis B charakterisiert. Diese CD4 T-Zellen produzieren ohne *ex vivo* Antigen-Restimulation TNF-α, aber nicht IFN-γ, IL-2 und MIP1-β ($CD4^{TNF\text{-}mono}$). Patienten mit einer akuten oder chronischen Hepatitis B wiesen im Vergleich zu gesunden, HBV-naiven Probanden und Probanden mit einer ausgeheilten HBV Infektion signifikant höhere Frequenzen dieser $CD4^{TNF\text{-}mono}$ T-Zellen auf. Erhöhte Frequenzen von $CD4^{TNF\text{-}mono}$ T-Zellen wurden auch bei Patienten mit einer persistierenden Hepatitis C Virus- (HCV) oder HIV-Monoinfektion, sowie bei Patienten mit einer HIV/HCV- oder HIV/HBV-Koinfektion nachgewiesen. Patienten mit einer chronischen Hepatitis B oder C unter IFN-α Therapie wiesen signifikant erhöhte $CD4^{TNF\text{-}mono}$ T-Zell-Frequenzen gegenüber nicht-therapierten Patienten auf. $CD4^{TNF\text{-}mono}$ T-Zellen sind antigenerfahrene Gedächtnis-T-Zellen, die überwiegend lösliches TNF-α produzieren. Die Zellen exprimieren keine T-Zell-Aktivierungs- oder *Exhaustion*-Marker und sind keine regulatorischen T-Zellen. Des Weiteren scheint es sich bei $CD4^{TNF\text{-}mono}$ T-Zellen nicht um HBV-spezifische T-Zellen zu handeln. $CD4^{TNF\text{-}mono}$ T-Zellen stellen eine bisher unbeschriebene T-Zellpopulation dar, die möglicherweise durch TNF-α vermittelte Induktion von Apoptose in Hepatozyten zur Immunpathogenese der chronischen Hepatitis B beiträgt.

Zusammenfassend liefert diese Arbeit wichtige neue Erkenntnisse für die Etablierung eines validen T-Zell-Monitorings zur Entwicklung und Evaluierung von T-Zell-modulierenden Immuntherapien gegen chronische Hepatitis B. Zusätzlich könnten $CD4^{TNF\text{-}mono}$ T-Zellen durch ihre potenziell leberschädigende Wirkung einen Angriffspunkt für Immuntherapien zur Behandlung der chronischen Hepatitis B darstellen.

# Summary

The human hepatitis B virus (HBV) is a non-cytopathic, enveloped DNA virus with a strong liver tropism. HBV infection is a global health burden as more than 780.000 people die each year as a consequence and more than 240 million people are currently chronically infected. Chronic HBV infection is associated with a progressive inflammation and injury of the liver (hepatitis B) which can lead to liver cirrhosis and hepatocellular carcinoma. The current standard therapies against chronic hepatitis B are not able to efficiently resolve HBV infection, hence new therapy approaches to treat this chronic disease are urgently needed. Since chronic hepatitis B is associated with impaired HBV-specific T-cell responses immunotherapeutic strategies that aim to restore functionality of antiviral T cells might be a promising strategy.

To develop new immunotherapies the identification of effective HBV-specific T-cell responses is relevant. An important strategy to identify effective HBV-specific T-cell responses is to monitor T cells in patients with self-limiting and chronic hepatitis B. Further, T-cell monitoring is used in clinical trials to prove efficacy of T-cell modulating therapies in patients with chronic hepatitis B. Therefore the present study dealt with the establishment of a sensitive, precise and reliable assay to monitor HBV-specific T-cell responses. *In vitro* expansion of peripheral blood mononuclear cells (PBMC) is commonly used prior to a T-cell monitoring to increase low numbers of HBV-specific T cells and enhance assay sensitivity. In order to evaluate the impact of a 10-day *in vitro* expansion on HBV-specific T cells, frequency and functionality of HBV surface- (HBs) and core- (HBc) specific CD4 and CD8 T cells *ex vivo* and after *in vitro* expansion was compared in 41 individuals with different HBV infection status (acute, chronic or resolved infection). T-cell reactivity was analysed by intracellular cytokine staining (IL-2, IFN-γ, TNF-α). *In vitro* expansion significantly increased the number of HBc-specific CD4/CD8 T cells, HBs-specific CD4 T cells but not HBs-specific CD8 T cells. Capacity of HBV-specific T cells to proliferate *in vitro* weakly correlated with the detected *ex vivo* frequency and was independent of the donor`s HBV infection status. Overall functional composition (cytokine profile) of expanded HBV-specific T cells differed to *ex vivo* detected HBV-specific T-cell responses. The proliferative capacity *in vitro* correlated with the degree of changes in functionality. No general increase of assay sensitivity was observed after *in vitro* expansion. In conclusion an *ex vivo* monitoring of HBV-specific T cells might assess the *in vivo* T-cell reactivity best and should thus be considered for future T-cell monitoring.

Additionally, a novel CD4 T-cell subpopulation was characterized in patients with chronic hepatitis B. In the absence of antigen restimulation *ex vivo* these CD4 T cells produce TNF-α but not IFN-γ, IL-2 and MIP1-β ($CD4^{TNF\text{-}mono}$). Patients suffering from acute or chronic

hepatitis B showed significantly higher frequencies of $CD4^{TNF\text{-}mono}$ T cells compared to healthy, HBV naïve individuals and individuals with resolved hepatitis B. $CD4^{TNF\text{-}mono}$ T cells were also detectable in elevated frequencies in patients with persistent hepatitis C virus (HCV) or HIV monoinfection and HIV/HCV or HIV/HBV coinfection. Treatment of chronic hepatitis B and C patients with pegylated IFN-α significantly increased frequency of $CD4^{TNF\text{-}mono}$ T cells compared to non-treatment. $CD4^{TNF\text{-}mono}$ T cells are antigen-experienced, memory T cells that mainly produce soluble TNF-α. The cells do not express T-cell activation or exhaustion markers and are not regulatory T cells. Further, $CD4^{TNF\text{-}mono}$ T cells seem not to be HBV-specific T cells. In conclusion $CD4^{TNF\text{-}mono}$ T cells might represent a not yet described bystander T-cell population which may contribute to immunopathogenesis of chronic hepatitis B by TNF-α mediated killing of hepatocytes.

In summary this work provides important knowledge for the development of a valid T-cell monitoring in order to establish and evaluate T-cell modulating therapies against chronic hepatitis B. Due to their potential role in liver damage $CD4^{TNF\text{-}mono}$ T cells might in addition represent a target for immunotherapies against chronic hepatitis B.

# Abbreviations

| | |
|---|---|
| aa | amino acid |
| Ag | antigen |
| ALT | alanine aminotransferase |
| APC | antigen presenting cell |
| BFA | Brefeldin A |
| bp | base pair |
| BSA | bovine serum albumin |
| cccDNA | covalently closed circular DNA |
| CD | cluster of differentiation |
| cDNA | complementary DNA |
| CTLA4 | cytotoxic T lymphocyte antigen-4 |
| CMV | cytomegalovirus |
| DC | dendritic cell |
| DMSO | dimethylsulfoxid |
| DNA, RNA | deoxy-, ribonucleic acid |
| EBV | Epstein-Barr virus |
| EDTA | ethylenediaminetetraacetic acid |
| FACS | fluorescence activated cell sorting |
| FEC | influenza (flu), Epstein-Barr, cytomegalovirus |
| Flu | influenza virus |
| FoxP3 | forkhead-box-protein 3 |
| FSC | forward scatter |
| HBs | HBV surface protein |
| HBc | HBV core protein |
| HBe | HBV e protein |
| HBV | hepatitis B virus |
| HCC | hepatocellular carcinoma |
| HCV | hepatitis C virus |
| HIV | human immunodeficiency virus |
| h | hours |

| | |
|---|---|
| HLA | human leukocyte antigen |
| HS | human serum |
| ICS | intracellular cytokine staining |
| IFN | interferon |
| Ig | immunoglobulin |
| IL | interleukin |
| kb | kilobase |
| l | liter |
| LCMV | lymphocytic choriomeningitis virus |
| M | molar |
| Mdn | Median |
| MHC | major histocompatibility complex |
| min | minute |
| MIP | macrophage inflammatory protein |
| MVA | modified vaccinia Ankara |
| NA | nucleot(s)ide analogue |
| NEAA | non-essential amino acids |
| NIR | LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit |
| NK cell | natural killer cell |
| NTCP | sodium taurocholate cotransporting polypeptide |
| ORF | open reading frame |
| PD1 | programmed death-1 |
| PBMC | peripheral blood mononuclear cells |
| Pen/Strep | penicillin/streptomycin |
| PEG-IFN-α | pegylated IFN-α |
| pgRNA | pregenomic RNA |
| PMA+I | phorbol-12-myristrate-13-acetate + Ionomycin |
| polyA | polyadenylation |
| PRR | pattern recognition receptor |
| rcDNA | relaxed circular DNA |
| resp. | respectively |

| | |
|---|---|
| SSC | side scatter |
| Tim3 | t-cell immunoglobulin- and mucin-domain-containing 3 |
| TLR | toll-like receptor |
| TNF | tumor necrosis factor |
| TRAIL | TNF-related apoptosis-inducing ligand |
| $T_{reg}$ | regulatory T cell |
| SVP | subviral particle |
| w/o | without |
| WHV | woodchuck hepatitis virus |

# 1 Introduction

## 1.1 Hepatitis B virus

### 1.1.1 Taxonomy and morphology

The human hepatitis B virus (HBV) belongs to the *Orthohepadnavirus* genus within the *Hepadnaviridae* family which are the smallest DNA containing, enveloped animal viruses known so far [1]. Hepadnaviruses all show very narrow host specificity, marked tropism for liver tissue and replication via an RNA intermediate by an encoded reverse transcriptase [2]. Since the viral polymerase lacks proofreading activity eight different genotypes of hepatitis B virus (A–H) have been evolved from which five (A-D and F) can be further divided in several subgenotypes. The different genotypes show at least 8% sequence diversity while sequence of subgenotypes differs at least 4% [3]. HBV genotypes influence course of disease as well as treatment response and are distinctly distributed worldwide with genotype A as the most prevalent in northwestern Europe and the USA, genotype B and C in Asia, and genotype D in Southern Europe and the Mediterranean basin (Figure 1.1) [2, 4, 5] .


**Figure 1.1: Geographical distribution of HBV genotypes A-H and subgenotypes [3].**

The infectious HBV particle, also called Dane particle, has a spherical structure of 42-44nm with an envelope containing three related surface antigens: HBsAg (small), M-protein (middle) and L-protein (large) (Figure 1.2A). All three surface proteins share the same C-terminal domain containing the HBsAg with the anti-HBs neutralizing domain, while M- and L-protein are N-terminally prolonged by a preS2 and latter also by a PreS1 domain. The viral nucleocapsid is formed by the core protein (HBcAg) that polymerizes to an icosahedral form

surrounding the HBV DNA and polymerase [1, 6]. In addition to infectious particle release, HBV infection is characterized by a $10^3$-$10^6$ fold increased secretion of spherical and filamentous non-infectious subviral particles (SVPs) into the blood of infected individuals (Figure 1.2B) [1]. These SVPs are exclusively formed by host lipids containing predominantly HBsAg and seems to negatively influence the host`s immune system [7, 8].

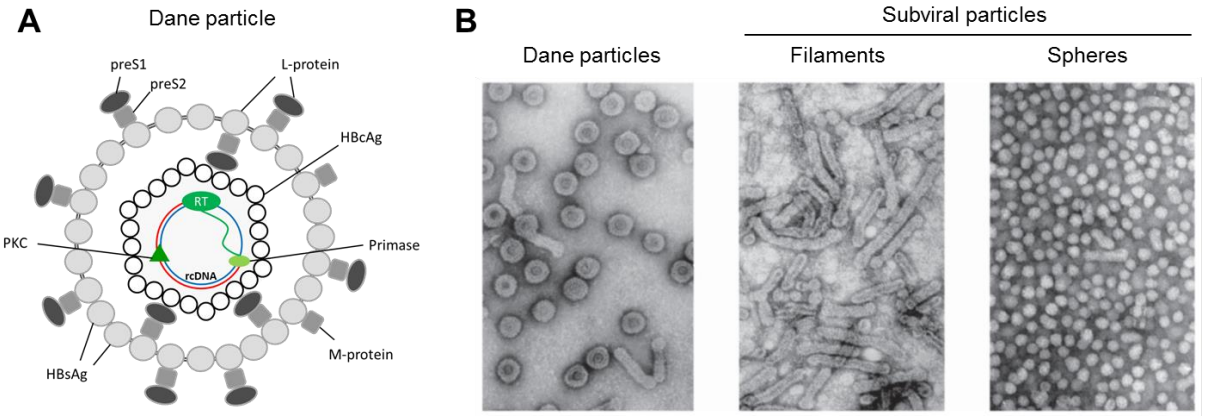



**Figure 1.2: Particle structures of HBV.** (A) Schematic view of an infectious HBV (Dane) particle [9]. The partially double stranded, relaxed circular DNA genome (rcDNA; red and blue line) is associated with the viral polymerase´s reverse transcription (RT) and primer domain (Primase) and the viral protein kinase C (PKC). 120 dimerized core proteins form the nucleocapsid incorporating the viral genome. Nucleocapsid is surrounded by an envelope consisting of host lipids and viral surface proteins (HBsAg (small), M-protein (middle), L-protein (large)). M- and L-protein are in comparison to HBsAg enlarged by a preS2 and latter also by a preS1 domain. (B) Electron microscopy pictures of infectious (Dane) and non-infectious, subviral HBV particles [3].

### 1.1.2 Genomic organization

Infectious HBV particles harbour a partially double stranded, relaxed circular (rc)DNA genome consisting of about 3200 nucleotides (Figure 1.3). The full-length minus strand is covalently linked with the viral polymerase´s reverse transcription (RT) domain at its 5`end while the 5' end of the incomplete plus strand is associated with an oligoribonucleotide serving as initiator of transcription (primase) [10]. After uptake of HBV into a hepatocyte and release of rcDNA into the nucleus, the viral polymerase is removed and plus-strand is completed by host components of the cellular replicating machinery leading to formation of the covalently closed circular DNA (cccDNA). From the cccDNA four different viral RNA molecules (0.7, 2.1, 2.4, 3.5 kb transcript) are transcribed and exported to the cytoplasm where they serve as mRNAs for protein translation and the 3.5 kb transcript in addition as template for HBV replication. The viral proteins are translated by using four different open reading frames (ORFs): preS/S, preC/C, X and P. The preS/S ORF encodes for the three different surface proteins, the preC/C ORF for the capsid and HBe protein (HBeAg), the X ORF for the X protein and the P ORF for the polymerase. HBeAg is a secreted accessory

protein that is not required for HBV replication but seems to suppress host´s immune response against the core protein [11, 12]. HBeAg levels in patient`s plasma are used as a surrogate marker for HBV replication [13]. In contrast HBx protein is a transactivator protein with oncogenic potential that is essential for productive HBV infection [14, 15].

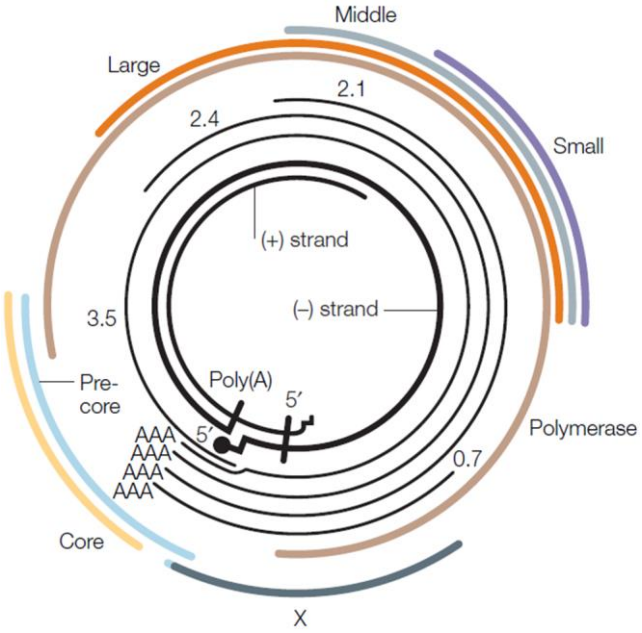



**Figure 1.3: Genomic structure of HBV [10].** The full-length minus (-) strand associated with polymerase`s RT domain protein at the 5´end and the incomplete plus (+) strand of the HBV genome are shown as the inner black circles. The surrounding thinner black lines represent the in length different RNA transcripts (0.7, 2.1, 2.4, 3.5 kB) terminating all near the polyadenylation site (Poly(A)). The outer colored lines indicate the translated HBV proteins including the small, middle and large surface proteins, polymerase protein, X protein as well as core and pre-core (HBe) protein.

### 1.1.3 HBV replication cycle

The first step in HBV infection is a cell-type unspecific initial attachment to heparin sulfate proteoglycans followed by binding to the sodium taurocholate cotransporting polypeptide (NTCP) that is predominantly expressed on hepatocytes (Figure 1.4) [16, 17]. On the viral site these interactions are mediated by the myristoylated preS1 domain of the HBV L-protein [18]. Upon NTCP binding HBV enters the cell presumably via endocytosis followed by the release of the uncoated nucleocapsid from endocytic vesicles into the cytoplasm [19]. Nucleocapsid is transported to the nucleus where the rcDNA genome is presumably via a nuclear pore complex released into the nucleus [20]. With the help of host components of the cellular replicating machinery viral polymerase is removed from the rcDNA and plus strand is completed leading to formation of a cccDNA molecule inside the nucleus [21]. cccDNA serves as transcription template for the four viral RNA molecules which are generated with 5´Cap and 3`poly(A) site by the cellular RNA polymerase II. Synthesized RNA molecules are exported into the cytoplasm where they are used for translation of viral proteins from four

different ORFs as described in 1.1.2. Synthesis of HBeAg and the surface proteins takes place at the endoplasmatic reticulum (ER) membrane. The largest RNA (pre-genomic) transcript can initiate assembly with core proteins and polymerase into immature nucleocapsids via a cis-acting element (epsilon) that contains a stem-loop structure. Within the nucleocapsid the polymerase mediates synthesis of the minus strand followed by degradation of the RNA template and DNA-dependent synthesis of the partial plus strand [6]. Mature nucleocapsids can either be recycled to amplify the cccDNA pool in the nucleus in order to ensure persistence of the HBV genome or acquire an envelope containing HBV surface proteins by budding into the endoplasmatic reticulum [6, 22]. Infectious HBV particles are finally released presumably through budding into multivesicular bodies [23]. In contrast non-infectious subviral particles and HBeAg seems to be secreted by an ER- and Golgi-dependent mechanism [24]. Due to continuous exchange of ER and plasma membrane it is likely that HBV surface proteins that are not associated with infectious or subviral particles can reach the cell surface.

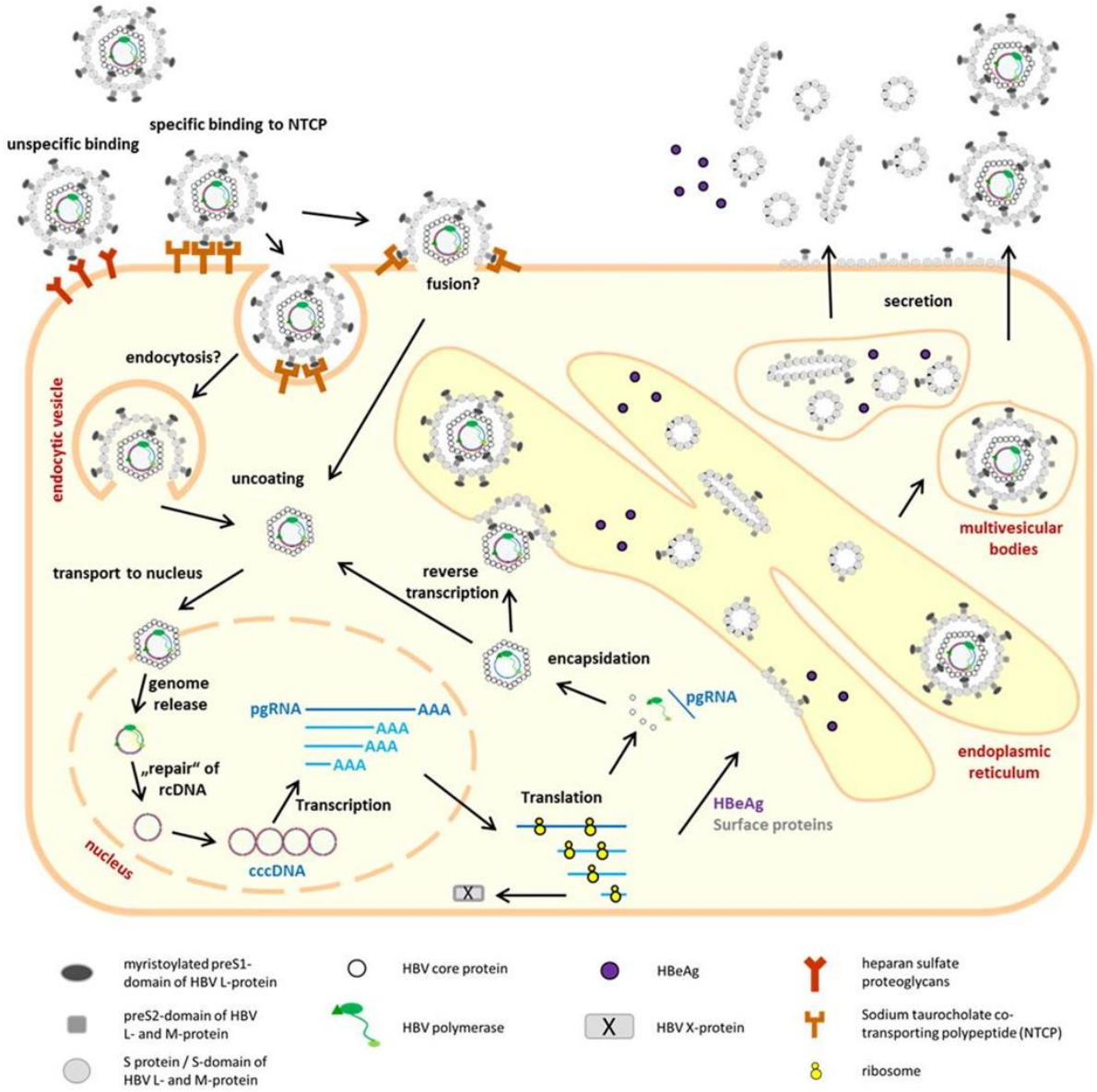



**Figure 1.4: HBV replication cycle [9].** After unspecific binding to heparin sulfate proteoglycans and specific binding to NTCP receptor, HBV enters into the hepatocyte and releases its nucleocapsid into the cytoplasm. Nucleocapsids transport the relaxed circular (rc)DNA genome into the nucleus where it is converted into a covalently closed circular DNA (cccDNA) molecule. cccDNA is used as transcription template for the four viral RNA molecules with different length that are exported into the cytoplasm where they are used as mRNAs for translation of viral proteins. HBeAg and surface proteins are synthesized at the endoplasmatic reticulum (ER). The longest RNA (pre-genomic; pg) transcript in addition serves as template for reverse transcription which takes place within immature nucleocapsids in the cytoplasm. Mature nucleocapsids are either recycled to fill up nuclear pool of cccDNA or enveloped by passing the ER until they are finally released through multivesicular bodies. HBeAg and subviral particles are secreted via ER and Golgi.

## 1.2 Hepatitis B virus infection

### 1.2.1 Epidemiology and transmission

HBV infection is a public health problem that can cause chronic liver inflammation (chronic hepatitis B) leading to a high risk for liver cirrhosis and hepatocellular carcinoma (HCC). More than 240 million people worldwide are chronically infected with HBV and approximately 780 000 people die each year as consequence of acute and chronic HBV infection [25]. Hepatitis B is most prevalent in sub-Saharan Africa and East Asia where 5–10% of the adult population is chronically infected (Figure 1.5). Additionally, a high amount of chronic carriers are found in the Amazon and the southern parts of eastern and central Europe. Approximately 2–5% of the population is chronically infected in the Middle East and the Indian subcontinent while less than 1% of the population in western Europe and North America is affected by chronic hepatitis B [25].

HBV is transmitted via blood and other body fluids of infected persons. In highly endemic areas mother to child transmission at birth and transmission from person to person in early childhood are the dominant routes of infection. In contrast HBV spreads predominantly by sexual transmission and use of contaminated needles in low endemic regions [25].

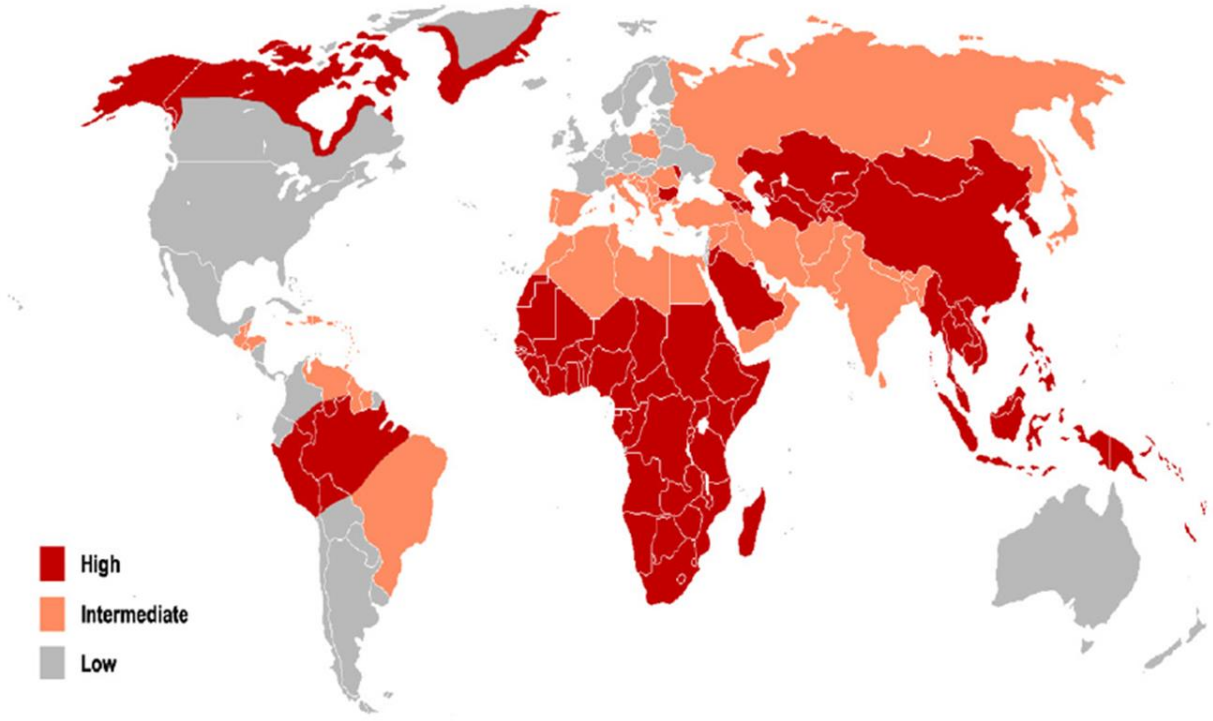



**Figure 1.5: Epidemiology of HBV infection [3].** Worldwide prevalence of HBV infection determined by HBsAg seroprevalence. High ≥8%, intermediate 2-7%, low ≤ 2%.

### 1.2.2 Natural course of HBV infection

Even though HBV is a non-cytopathic virus it leads to liver disease including acute and chronic viral hepatitis as well as liver cirrhosis and hepatocellular carcinoma as consequence of chronic infection. Hepatitis B pathogenesis and outcome is strongly mediated by the host immune response, mainly T cells, however, molecular mechanisms determining HBV persistence and pathogenesis are still poorly defined [1].

In immunocompetent adults HBV infection results in 95% in a self-limited, transient liver disease associated with immune mediated clearance of HBV [26]. Typically acute HBV infection starts with an incubation phase of about 30 days without detectable HBV markers followed by an exponential increase of HBV DNA and HBsAg in the serum (Figure 1.6A) [3]. 6 weeks after infection, before onset of the acute disease, HBV DNA, HBeAg and HBsAg titers reach their peak. With the onset of clinical symptoms HBV core antigen (HBcAg)-specific IgM antibodies appear and irrespective of the outcome of infection HBcAg-specific IgG antibodies persists lifelong. Approximately 8–12 weeks after infection, raising serum alanine aminotransferase (ALT) levels are observed indicating T cell mediated killing of infected hepatocytes [10]. Besides cytolytic killing also a non-cytolytic, cytokine mediated clearance of HBV from infected hepatocytes seems to occur during acute HBV infection since most of HBV DNA in serum and liver of chimpanzees is already cleared before ALT levels peak [27]. HBeAg and HBsAg disappear from circulation with the development of HBeAg- and HBsAg-specific antibodies which preserve lifelong protective immunity. Disappearance of HBsAg is considered as sign of resolution, however, cccDNA persists in a small number of hepatocytes under control of humoral and cellular immune responses bearing the risk of virus reactivation in case of immunosuppression [10]. In some cases acute infection follows a fulminant course which is often associated with death due to liver failure [28].

In contrast to HBV infection during adulthood, vertical transmission from mothers to their children or horizontal transmission during early childhood leads in 90% to chronic infection defined as detectable circulating HBsAg for more than 6 months. The course of chronic hepatitis B is generally described as occurring in four different phases based on serum ALT and HBsAg levels, HBeAg serostatus and HBV DNA serum titer (Figure 1.6B) [29]. Nevertheless not all individuals with chronic hepatitis B show this sequential course of infection [30]. The first phase is characterized by high levels of serum HBV DNA, HBsAg and HBeAg while ALT levels and histological liver profile are normal although strong HBV-specific T-cell responses are observed [31]. This so called immunotolerant or better low inflammatory activity phase is particularly observed in children and young adults after vertical transmission and can last for more than 20 years [1, 31]. In contrast chronic hepatitis B acquired during

adulthood seems to miss this first phase and instead directly enter into the second phase. The second so called immunoactive phase is characterized by HBeAg positivity, fluctuating HBV DNA levels, lower levels of HBsAg, elevated serum ALT levels and histological damage why most infections are diagnosed in this phase [10]. At this stage immunoactive phase can progress to liver cirrhosis or alternatively, which might be the case in 80-90% of individuals, to a low replicative phase which is associated with development of HBeAg-specific antibodies and clearance of circulating HBeAg [31]. Typically these HBsAg positive patients show low/undetectable serum HBV DNA and normalizing ALT levels associated with slow disease progression. As HBeAg seroconversion predicts sustained multi-log reduction in HBV DNA, a decrease in liver damage and an improved prognosis, it is used as an important therapeutic endpoint in the management of HBeAg-positive chronic hepatitis B [1, 32]. Most patients remain in the low replicative phase which might last for life and about 40% of them are able to spontaneously clear HBV infection reflected by HBsAg seroconversion within 25 years [31]. However, about 15% of patients might experience spontaneous HBV reactivation associated with increased hepatitis activity and ALT flares (high replicative phase) [31, 33]. Mutations in the promoter region of the gene encoding HBcAg might be one reason for the reactivated HBV replication [10]. HBeAg-negative chronic hepatitis B cases has been reported as the most common type of hepatitis B, especially in mediterran Europe and Africa [34]. As consequence of chronic hepatitis B the life time risk to develop liver cirrhosis and HCC is about 15-40% [31].

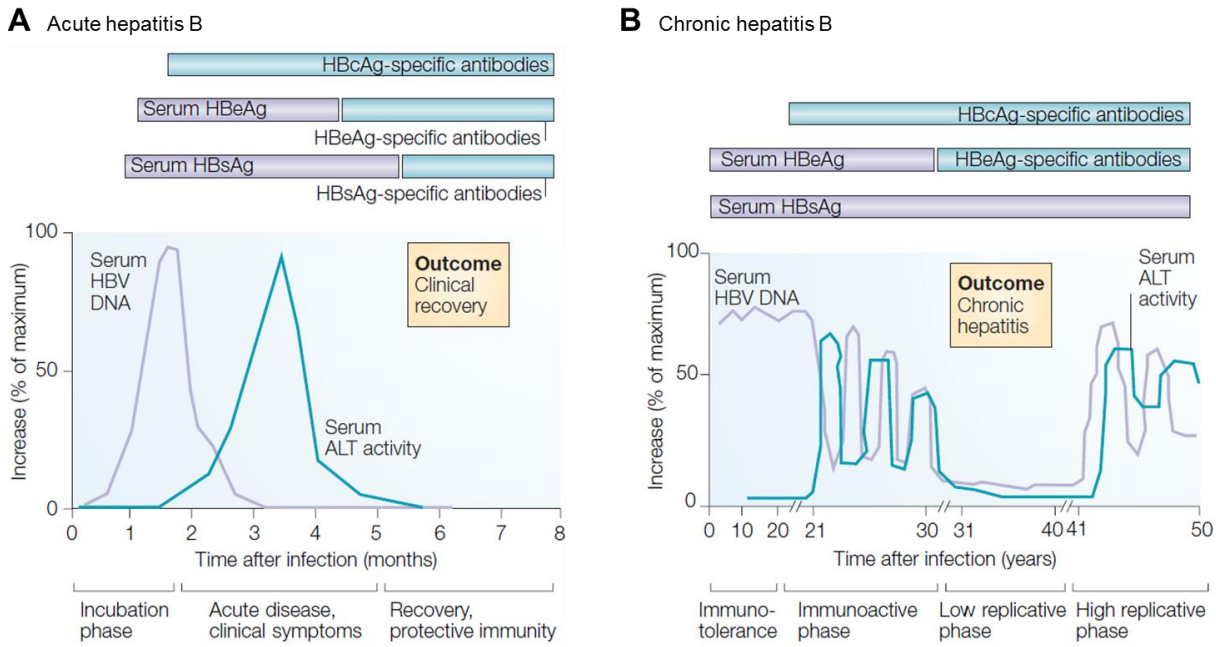



**Figure 1.6: Natural course of acute and chronic hepatitis B [10].** (A) Schematic view of the immune response in horizontally transmitted, acute hepatitis B. After initial strong replication HBV is resolved by host´s immune response. Clinical recovery is associated with lifelong, protective immunity consisting of neutralizing HBsAg-specific antibodies and HBV-specific T cells. (B) Schematic view of the immune response in vertically transmitted chronic hepatitis B. The course of disease is

characterized by several phases of variable length: the immunotolerant, immunoactive, low replicative and high replicative phase. Further details are described in the main text. HBc/e/sAg: HBV core/e/surface antigen; ALT: Alanine transaminase

## 1.3 Immune responses against HBV

### 1.3.1 Innate immune response

The innate immune system represents the first line of defense against viruses and additionally, plays an important role in the induction of adaptive immune responses. Innate immune cells recognize viruses by pattern recognition receptors (PRRs) like Toll-like receptors (TLR) which bind to pathogen/danger-associated molecular patterns. Activation of innate immune cells lead to an antiviral state mainly associated with production of type I Interferons (IFNs), killing of infected cells by natural killer (NK) cells and production of pro-inflammatory cytokines and chemokines that induce maturation and recruitment of adaptive immune cells [35, 36].

Due to restricted availability of *in vitro* and *in vivo* infection models as well as patient samples at early stages of infection the innate immune response in HBV infection, in contrast to the adaptive system, is not very well described. However, studies in patients and chimpanzees revealed lacking type I IFN and proinflammatory cytokine induction in acute HBV infection indicating that HBV evades innate immune recognition (stealth virus) [37, 38]. Conversely, *in vitro* studies showed that HBV infection of hepatocytes induces a type I IFN response that is able to limit spread of HBV [39-41]. Further HBV infection of human hepatocytes in chimeric mice resulted in moderately activated IFN-stimulated genes [1, 42]. Another observation supporting the hypothesis that HBV does not completely evade innate immune response is that primary human liver cells produce interleukin (IL)-6 upon HBV infection which was proposed to control early viral replication, limit adaptive immune responses and prevent hepatocyte death after HBV infection [36, 43]. Although the published data concerning HBV induced innate immune activation are controversial, they suggest that HBV seems not to be a complete stealth virus but is recognized by the innate immune system very early during infection in humans [26]. Numerous studies to elucidate the role of NK cells in HBV infection have also been performed as these cells account for up to 30–40% of the resident intrahepatic lymphocytes [44]. NK cells can be antigen-independently activated by interaction with inhibitory and activating receptors or cytokines like IL-12, IL-15 and IL-18 [45]. In mice activated NK cells can inhibit HBV replication by killing of HBV-infected hepatocytes, mainly via the TNF-related apoptosis-inducing ligand (TRAIL), and production of direct antiviral cytokines (interferon (IFN)-γ and tumor necrosis factor (TNF)-α) and immune cell recruiting chemokines [46, 47]. Studies of patients in the preclinical phase and in a follow-up until

resolution of HBV infection revealed that activation and effector functions are suppressed during peak of viremia which might be due to increased IL-10 levels in the early phase [36, 48]. In woodchucks induction of a gene which is related to NK-cell activation was observed immediately after infection with woodchuck hepatitis virus (WHV) indicating that NK cells might contribute to HBV control in the earliest incubation phase of infection [49, 50]. NK cells in patients with chronic hepatitis B upregulate TRAIL receptor and show increased cytotoxic potential associated with liver injury while they seem to have an impaired capacity to produce IFN-γ [46, 51, 52]. Overall data about the role of NK cells in HBV persistence and the mechanisms that regulate NK-cell effector functions are controversial und further studies are needed to gain insight in this topic.

### 1.3.2 Adaptive immune response

While there is still a debate about the role of innate immune responses in early control of HBV, it is generally accepted that strong and polyfunctional virus-specific T cells are essential for control of HBV infection [50]. As mentioned above CD4 and CD8 T-cell responses are detectable 8-12 weeks after infection in response to exponentially increasing HBV DNA levels that reach a peak 6 weeks after infection (Figure 1.6A) [10]. Due to undetectable HBV markers in the initial incubation phase, T cells are not sufficiently stimulated at this time. Thus appearance of T-cell responses is delayed relative to the time point of infection compared to other viral infections [26]. T-cell responses are followed by humoral immune responses detectable 10-15 weeks after infection which are an important component of protective immunity against HBV [53]. Recovery from hepatitis B results in life-long protective immunity mediated by neutralizing HBsAg-specific antibodies and by HBV-specific CD4 and CD8 T cells [10].

#### 1.3.2.1 CD4 T-cell responses

HBV-specific CD4 T cells are crucial for control of HBV infection as depletion of CD4 T cells prior infection causes persistent infection with minimal immunopathology in chimpanzees [54]. In contrast CD4 T-cell depletion at the peak of HBV infection had no effect on viral clearance and liver disease indicating that CD4 T cells are rather crucial for induction of efficient cytotoxic CD8 T-cell responses and T cell-dependent B-cell responses than having major direct antiviral effects [36, 54]. Patients with acute HBV infection show almost all vigorous peripheral CD4 T-cell responses against multiple epitopes within the HBcAg [55-57]. Most of these CD4 T-cell responses are also directed against HBeAg due to strongly overlapping protein sequences. HBsAg-specific CD4 T cells are detected in lower frequencies compared to HBcAg-specific CD4 T cells in these patients [26, 56, 58]. However, this cannot be attributable to a general weak immunogenicity of HBsAg since vaccination with subviral particles induces a strong and oligoclonal HBsAg-specific T-cell response in

healthy individuals [59]. Beside the dominating HBcAg-specific CD4 T-cell responses, also CD4 T cells directed against epitopes within the HBV polymerase and the regulatory HBx protein seem to play a role in clearance of HBV infection [26, 60]. HBV-specific CD4 T cells seem to contribute to liver damage in acute HBV infection as HBcAg-specific CD4 T cells mainly produce the Th1-type cytokines TNF-α and IFN-γ [26]. After HBV resolution the number of HBcAg-specific CD4 T cells declines but they are still detectable for decades after clinical recovery [61, 62].

In contrast to patients with acute hepatitis B, patients with chronic hepatitis B show weaker narrowly focused epitope-specific CD4 T-cell responses [55]. A polyclonal HBV-specific CD8 T-cell response in the absence of a corresponding CD4 T-cell response after HBV infection has been associated with chronicity indicating again that CD4 T cells are crucial for the maturation of efficient HBV-specific CD8 T cells [63]. Hence restoration of CD4 T-cell function might be an important target for immunotherapies against chronic hepatitis B. Thus further understanding of the mechanisms behind CD4 T-cell failure in chronic hepatitis B is needed. Currently only limited data about differentiation, maturation and function of CD4 T cells during the natural course of HBV infection are available due to the low frequency of circulating HBV-specific T cells [26].

#### 1.3.2.2 CD8 T-cell responses

In contrast to HBV-specific CD4 T cells, CD8 T cells directed against HBV were extensively studied. HBV-specific CD8 T-cell responses are crucial for control of HBV infection. Strong and multi-specific HBV-specific CD8 T-cell responses are associated with HBV clearance in acute hepatitis B [64]. Depletion of CD8 T cells in HBV infected chimpanzees further confirmed the central antiviral role of CD8 T cells as HBV titers remained at high levels in the absence of CD8 T cells [54]. However, there is still a debate about the mechanisms CD8 T cells mediate viral control [65]. Studies in chimpanzees, HBV-transgenic mice and in a cell culture model have shown that IFN-γ and TNF-α mediated non-cytolytic mechanisms contribute to viral control by CD8 T cells [27, 66, 67]. In addition also cytolytic effects seem to contribute to viral control by CD8 T cells [68, 69]. Further it was reported that HBV-specific CD8 T cells produce macrophage inflammatory protein 1-β (MIP1-β), a chemokine which might attract further immune cells that contribute to antiviral immune response [70].

In patients with acute hepatitis B CD8 T cells directed against several epitopes within HBcAg, HBsAg, HBV polymerase and HBx protein have been identified [26]. These CD8 T-cell responses remain detectable for decades after HBV resolution [71-73]. In contrast HBV-specific CD8 T cells are barely detectable in patients with chronic hepatitis B [15, 55, 74-76]. Thereby quantity and quality of HBV-specific CD8 T-cell responses inversely correlates with HBV replication. Specifically, CD8 T cells directed against immunodominant HBV epitopes,

like $HBcAg_{18–27}$ in HLA-A2 positive donors, have been shown to be hardly detectable if viral load exceeds $10^7$ copies/ml [15]. One explanation for low frequency of detectable HBV-specific T cells in chronic hepatitis B might be progressive loss of T-cell function (exhaustion) due to high levels of persisting viral antigens as it has been already shown for chronic murine lymphocytic choriomeningitis virus (LCMV) infection in mice [77-79]. HBV-derived peptides are constantly presented on major histocompatibility complex (MHC) molecules and additionally, circulating HBsAg and HBeAg might induce persistent T-cell stimulation [65]. It has been shown that HBeAg levels influence reactivity of virus-specific CD8 T cells and moreover that HBsAg seroconversion restores more effectively CD8 T-cell responses than HBV viral load reduction alone [88, 89]. Exhaustion of T cells leads gradually to the failure of cytokine production (often in a hierarchical manner; IL-2 < TNF-α < IFN-γ), loss of proliferative capacity upon antigenic stimulation and a substantial loss of cytotoxic activity (Figure 1.7) [80]. In case of prolonged and/or severe infection exhausted T cells can finally be completely eliminated leading to loss of HBV-specific T cells. Dysfunction of HBV-specific CD8 T cells in chronic hepatitis B is associated with elevated expression of inhibitory receptors like programmed cell death 1 (PD1), cytotoxic T lymphocyte antigen 4 (CTLA4), Tim3 and 2B4 [76, 81-85]. The amount and diversity of expressed inhibitory receptors thereby correlates with severity of T-cell exhaustion. *In vitro* studies have been shown that blockade of the mentioned inhibitory pathways is at least partially able to restore function of HBV-specific CD8 T cells [76, 81, 82, 84, 85]. The relevance of PD1 pathway in T-cell dysfunction observed in patients with chronic hepatitis B was further confirmed by blockade experiments in HBV mouse and woodchuck model [86, 87]. As mentioned in 1.3.2.1 also the lack of CD4 T-cell help accounts for defective CD8 T-cell function in chronic hepatitis B. The mechanisms underlying T-cell exhaustion during chronic HBV infection are still not fully understood however, taken together severity of T-cell exhaustion correlates with upregulation of inhibitory receptors, high viral or antigen load, loss of CD4 T-cell help and prolonged infection (Figure 1.7).

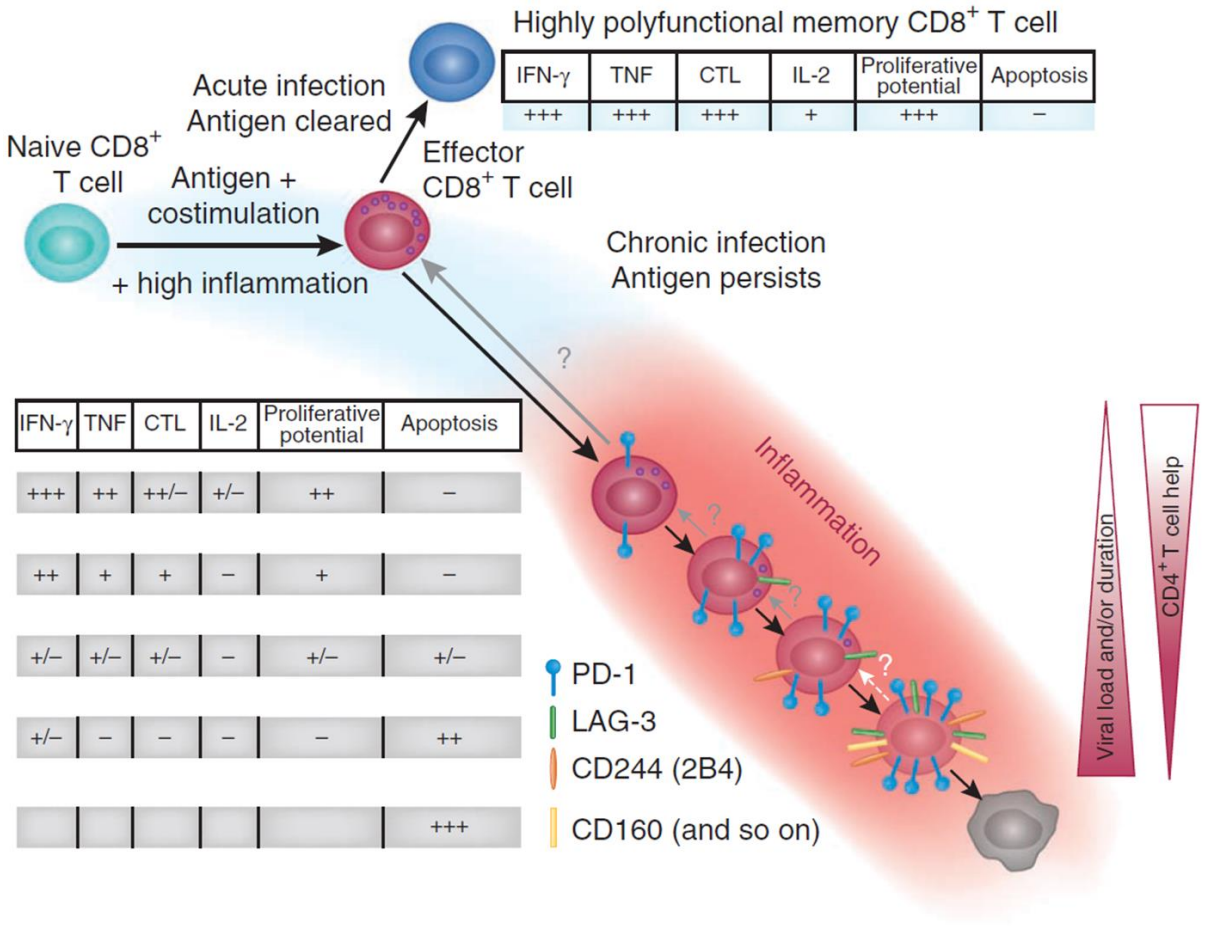



**Figure 1.7: Hierarchical T-cell exhaustion in chronic hepatitis B [80].** Initial HBV infection induces priming of naïve HBV-specific CD8 T cells by antigen, costimulation and inflammation leading to differentiation of these cells into effector T cells. After resolution a subset of functional HBV-specific memory CD8 T cells persists which are able to produce many cytokines in parallel (such as IFN-γ, TNF-α and IL-2) and have cytolytic and proliferative capacity (top). During chronic infection (bottom), antigen persists leading to T-cell dysfunction by loss of effector functions in a hierarchical manner. In parallel with T-cell exhaustion a progressive increase in the amount and diversity of inhibitory receptors occurs. In case of prolonged and/or severe infection exhausted T cells can finally be completely eliminated leading to loss of HBV-specific T cells. Severity of T-cell exhaustion correlates with upregulation of inhibitory receptors, high viral or antigen load, loss of CD4 T-cell help and prolonged infection. Properties of CD8 T cells are presented on a scale from high (+++) to low (-); CTL: cytotoxic potential

Beside T-cell exhaustion, T-cell failure in chronic hepatitis B might be mediated by depletion of HBV-specific CD8 T cells as Bim - a pro-apoptotic member of the Bcl-2 superfamily – has been shown to be upregulated in HBV-specific CD8 T cells obtained from patients with chronic HBV infection [88-90]. Further downstream blockade of Bim could restore oligoclonal functional responses directly *ex vivo* in these donors. It has been hypothesized that this pro-apoptotic status might be induced by tolerogenic activation via antigen-presenting cells (APCs) in the liver [88]. To maintain functional HBV-specific CD8 T-cell responses a balance between co-inhibitory and co-stimulatory signals during intrahepatic activation is crucial. It has been shown that expression of the co-inhibitory receptor PD1 ligand is upregulated on

intrahepatic cells in chronic HBV infection [91]. In line with these observations CD8 T cells from patients with chronic hepatitis B showing high viral loads have a severe dysfunction which is more pronounced in the liver than in the blood [81, 92]. Further it has been reported that the lack of arginine in the liver mediated by increased intrahepatic arginase levels could induce functional silencing of HBV-specific CD8 T cells by downregulation of the CD3ζ-chain [93-95]. Moreover HBV-specific CD8 T cells might be insufficiently primed by APCs and therefore not expand upon antigen-encounter as impairment of DCs was described in chronically HBV infected patients [96-99].

#### 1.3.2.3 Regulatory T cells

$FoxP3^{+}CD25^{+}CD127^{low}CD4^{+}$ T cells resemble the classical $T_{regs}$ which are able to suppress virus-specific immune responses via direct cell-cell contact or by secretion of immunosuppressive cytokines such as IL-10 and transforming growth factor (TGF)-β. It was shown that $T_{regs}$ can suppress HBV-specific T-cell functions however, their role in persistence of HBV infection remains elusive due to contradictory data [36, 100-102]. Several studies described increased frequencies of peripheral $T_{regs}$ in patients with chronic hepatitis B whereas others showed no correlation [103, 104] [102, 105]. *In vitro* $T_{reg}$ depletion led to an increased function of HBV-specific T cells from patients with chronic and acute hepatitis B and additionally, $T_{reg}$ frequency inversely correlated with ALT levels [102, 103]. These data indicate that $T_{regs}$ have anti-inflammatory effects without having major effects on HBV persistence [36].

#### 1.3.2.4 Humoral immune responses

Humoral immune responses are necessary to establish protective immunity against HBV however, it is not clear to what extent they contribute to control of chronic HBV infection. Antibodies against HBsAg (anti-HBs) completely prevent infection and can be observed in individuals vaccinated against HBV and patients recovered from HBV infection. Further anti-HBs seroconversion is associated with resolution of HBV infection. Anti-HBs is undetectable in the serum of chronic HBV carriers but at least 0.5-0.8% of patients with chronic hepatitis B in the low replicative phase (see Figure 1.6B) are able to seroconvert per year [106]. Antibodies against HBV core protein (anti-HBc) in contrast are not protective and can already be detected early during infection independent from the outcome of infection. HBeAg-specific antibodies (anti-HBe) which can be detected late during infection are associated with favorable disease outcome. In patients with chronic hepatitis B anti-HBe seroconversion initiates the low replicative phase of HBV.

### 1.3.3 Prophylaxis and therapy

In 1986, the first recombinant HBV vaccine consisting of yeast-derived HBsAg was approved [107]. Protective levels of anti-HBs persisting for at least 20 years can be achieved in more than 95% of infants, children, and young adults by complete immunization schedule [25, 107]. The recommendation of the WHO to integrate HBV vaccination into all national immunization programs led to a global coverage of complete HBV vaccination of 79% in 2012 [25]. In association with this worldwide HBsAg prevalence also remarkably dropped. Further anti-HBs immunoglobulins are effectively used as post-exposure prophylaxis in newborns of HBV-infected women and health care workers as well as to prevent HBV reactivation after liver transplantation [33, 107]. However, HBV remains a major health problem worldwide. To finally eradicate HBV vaccine coverage rate has to be further increased especially in highly endemic low-income countries [107].

Currently two therapeutic approaches are available to suppress HBV replication: antiviral agents (nucleos(t)ide analogues, NAs) and immune modulating therapies (IFN-α or pegylated (PEG)-IFN-α). Several NAs (lamivudine, adefovir, entecavir, telbivudine, and tenofovir) have been approved and are used as standard treatment for chronic hepatitis B. All of them interfere with HBV DNA replication as they target HBV polymerase. Treatment with NAs lead to a long lasting, strong suppression of HBV as well as restoration of functional impaired DC and HBV-specific T cells [76, 108, 109]. However, restoration of T-cell function seems to be only transiently [110]. Costs for this therapy are very high and patients have to be treated for a long time to achieve HBeAg or HBsAg seroconversion [111]. Anti-HBs seroconversion rate, however, is very low under NA treatment [112]. Treatment cessation leads to a high risk to reactivate HBV replication and HBeAg expression [113].

IFN-α or PEG-IFN-α act on the one hand as stimulator of the host´s immune system and on the other hand as a direct antiviral. Compared to NAs IFN-α and PEG-IFN-α show: (I) defined treatment duration (48 weeks), (II) no induction of resistances and (III) higher rate of HBeAg and HBsAg seroconversion. It has been shown that PEG-IFN-α treatment does not significantly reconstitute HBV-specific effector T-cell responses but might restore function of memory T cells in patients with chronic hepatitis by downregulation of inhibitory receptors in parallel with upregulation of effector molecules [114]. Further it was recently shown that IFN-α treatment of HBV infected hepatocytes *in vitro* lead to a specific and not hepatotoxic degradation of cccDNA [115]. However, treatment with IFN-α is limited as it is expensive, needs subcutaneous injection, is only effective in 30–40% of chronic hepatitis B patients and has several contraindications as well as significant side effects [116].

Treatment response rates of the current antiviral therapies are not satisfactory therefore several studies were performed to analyse efficacy of a combination therapy. Some data indicate that IFN-α and NA combination therapy is more effective compared to IFN-α and NA monotherapy like lamivudine [117, 118]. In contrast others showed no additional benefit of combination therapy compared to PEG-IFN-α monotherapy [119, 120]. Hence it is still not clear whether combination therapy of two NAs or NAs and IFN-α are superior to monotherapy. Possibly combination of NAs or IFN-α with other immunotherapies might be beneficial, hence there is a strong need for development of new immune-based therapies against chronic hepatitis B [116].

### 1.3.4 New immune-based therapy approaches

Due to the limitations of current antiviral therapies against chronic hepatitis B there is a strong need for new therapy approaches to address this major health problem. Approaches that aim to stimulate antiviral innate and adaptive immune responses in patients with chronic hepatitis B might be promising. TLR-agonists are able to stimulate innate immune responses in HBV-infected hepatocytes as it was shown that TLR activation can inhibit HBV replication in HBV-transfected hepatoma cell lines and in HBV-transgenic mice (Figure 1.8) [40, 121]. In addition efficacy of TLR-7 agonization could recently be shown in woodchucks and chimpanzees [36]. Another target might be intrahepatic NK cells which could be functionally restored by blockade of inhibitory cytokines such as IL-10 and TGF-β or directly activated using cytokines (IL-12, IL-18 or IFN-α) or TLR agonists [36, 122, 123]. T-cell receptor-like antibodies specifically targeting HBV-infected hepatocytes might be useful as a vehicle for drugs such as IFN-α to increase efficacy but limit side effects [124].

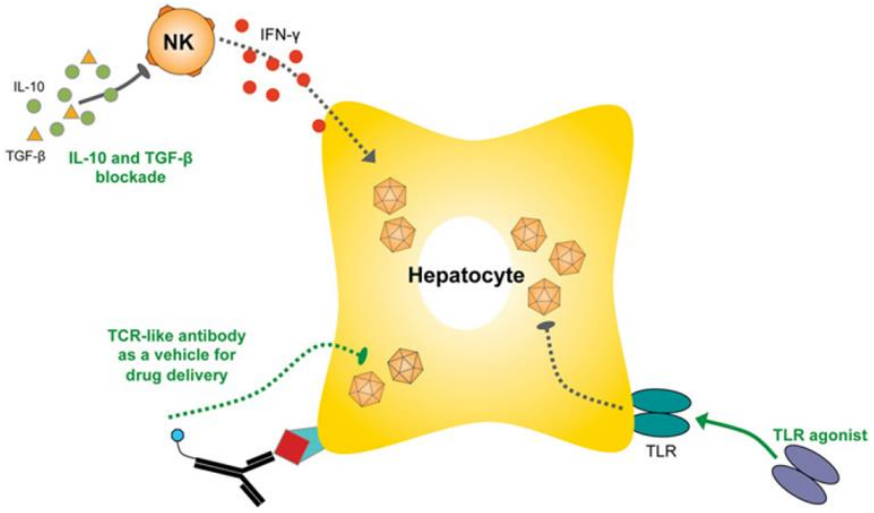



**Figure 1.8: Possible strategies to modulate innate immune responses in chronic hepatitis B [36].** TLR agonists are a promising immunotherapeutic approach to stimulate innate immune responses in HBV-infected hepatocytes. TCR-like antibodies might be useful for targeted delivery of antiviral drugs such as IFN-α to infected cells to increase efficacy but limit side effects. Further blockade of inhibitory cytokines such as IL-10 or TGF-β could reconstitute NK-cell function and thereby increase elimination of HBV-infected hepatocytes.

Beside stimulation of innate immune responses it might be also very promising to restore function of HBV-specific CD4 and CD8 T cells. One strategy might be the downstream blockade of the pro-apoptotic protein Bim which as in 1.3.2.2 discussed might be involved in impaired HBV-specific T-cell responses in chronic hepatitis B (Figure 1.9). As the inhibitory receptor PD1 is upregulated on dysfunctional HBV-specific T cells blockade of this receptor might be a potential target for immunotherapy in chronic hepatitis B (see 1.3.2.2). In HBV-transgenic mice treatment with blocking antibodies against PD1 ligand 1 significantly increased the number of IFN-γ positive CD8 T cells in the liver and delayed suppression of these cells [86]. Since PD1 blockade was already successfully tested in a clinical trial for cancer treatment, this approach is especially encouraging [125]. Taken into account that dysfunctional HBV-specific T cells express a combination of inhibitory receptors, it might be useful to modulate several co-inhibitory and costimulatory pathways including, for example, 2B4 and CTLA4 [36, 82, 85]. Further blockade of inhibitory cytokines such as IL-10 or TGF-β could reconstitute T-cell function and thereby increase elimination of HBV-infected hepatocytes. Beside the already described approaches also several strategies for a therapeutic vaccination in chronic hepatitis B have been developed and some of them seem to be promising [126]. Therapeutic vaccination strategies aim to eliminate HBV infection by stimulating the host's immune response for example by application of HBV peptides, proteins or DNA. Recently it could be shown that a therapeutic vaccination consisting of an HBc and HBs protein prime followed by a boost with Modified Vaccinia Ankara (MVA)-vectors encoding for HBc and HBs could induce functional HBV-specific T cells and anti-HBs in HBV-transgenic mice [9]. Another promising approach might be the use of functional engineered HBV-specific T cells by chimeric antigen or T-cell receptor gene transfer [127, 128].

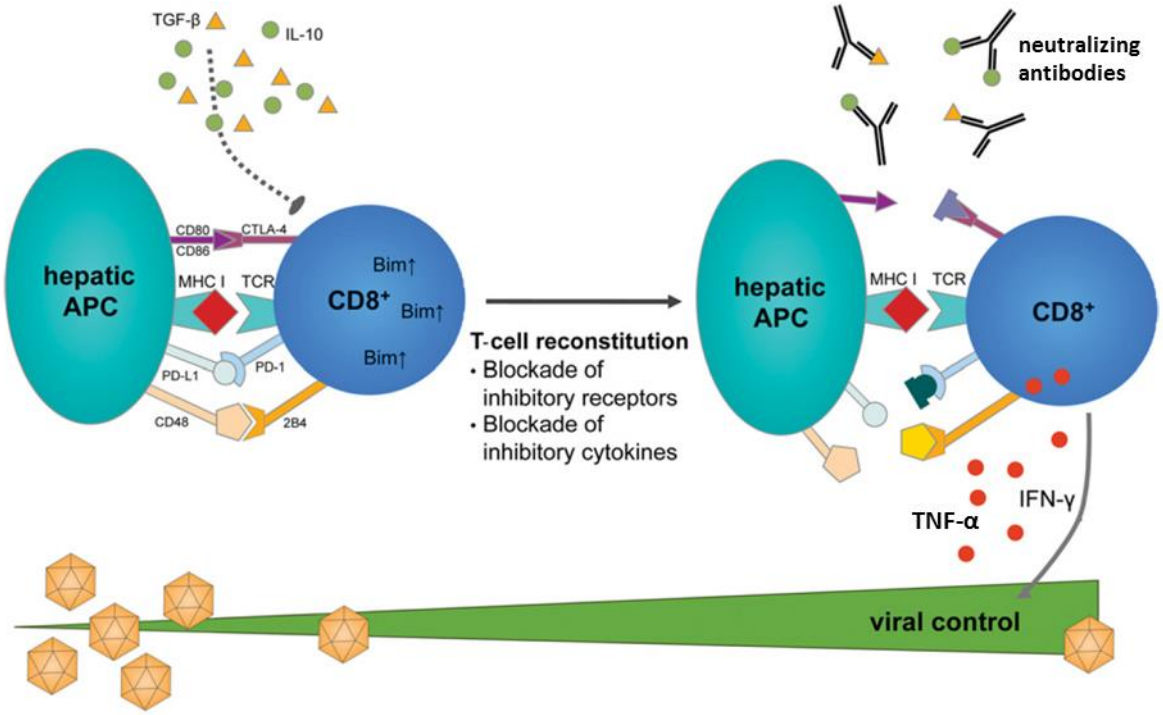



**Figure 1.9: Possible strategies to modulate adaptive immune responses in chronic hepatitis B adapted from [36].** Blockade of inhibitory receptors such as PD1 or 2B4 and cytokines such as TGF-β or IL-10 are promising approaches to restore CD8 T-cell function and increase elimination of HBV-infected hepatocytes.

## 1.4 T-cell monitoring

Potent immune-based therapies against chronic hepatitis B need to induce effective HBV-specific T-cell responses. Monitoring of HBV-specific T cells in patients with self-limiting and chronic hepatitis B is an important strategy to identify effective T-cell responses. Further, T-cell monitoring is an important primary endpoint in clinical trials that aim to prove efficacy of T-cell inducing therapies in patients with chronic hepatitis B. Hence methods to analyse quantity and functional profile of HBV-specific T cells in a sensitive, precise and robust manner are essential. T-cell responses can be monitored by a variety of assays (e.g. intracellular cytokine staining (ICS), enzyme-linked immunospot (ELISpot) and multimer staining) using different experimental protocols (e.g. monitoring *ex vivo* and after *in vitro* expansion). There are no internationally agreed protocols for assay performance and data analysis and common reference samples are lacking. Therefore data generated from different laboratories are difficult to compare and interpret. Hence there is a strong need for standardization and validation of immunoassays in international proficiency panels prior to their use in clinical trials.

## 1.5 Aims and objectives

As current standard therapies against chronic hepatitis B are unable to eradicate HBV, there is a strong need for innovative treatment strategies. Multifunctional, polyclonal HBV-specific T cells have been shown to be crucial for resolution of HBV infection. Therefore immunotherapies to restore dysfunction of HBV-specific CD4 and CD8 T cells in chronic hepatitis B might be a promising strategy. To identify signatures of effective HBV-specific T-cell responses in order to establish and evaluate new immunotherapies against chronic hepatitis B, sensitive, reliable and inter-laboratory comparable immunoassays are required. Polychromatic ICS is a flow cytometry-based method commonly used to monitor quantity and functional phenotype of antigen-specific T-cell responses in parallel [129]. T-cell analysis by polychromatic ICS requires standardized experimental protocols, as several parameters in the experimental procedure can impact marker expression of antigen-specific T cells [130-133]. *In vitro* expansion of peripheral blood mononuclear cells (PBMC) for up to 14 days is commonly used prior to a HBV-specific T-cell monitoring to increase assay sensitivity [134]. However, *in vitro* expansion of PBMC might change the HBV-specific T-cell population due to preferential expansion of particular T-cell clones which may not fully reflect T-cell reactivity *in vivo*. Therefore the first aim of this study was to establish a protocol for *in vitro* expansion of HBV-specific T cells and further analyse the impact of *in vitro* PBMC expansion on quantity and quality of HBV-specific CD4 and CD8 T cells.

*Ex vivo* T-cell monitoring in patients with chronic hepatitis B revealed a yet undescribed monofunctional CD4 T-cell population which does not produce IL-2, IFN-γ and MIP1-β but TNF-α ($CD4^{TNF\text{-}mono}$) without antigen restimulation *ex vivo* [70]. TNF mediated liver damage is an import mechanism of progressive liver damage in chronic hepatitis B [135, 136]. TNF-α is also able to negatively regulate CD4 T-cell responses and $T_{regs}$ [137] [138]. Therefore the second aim of this study was a detailed profiling of the $CD4^{TNF\text{-}mono}$ T-cell population to determine the potential role of these cells in immunopathogenesis of chronic hepatitis B. For this purpose two different polychromatic flow-cytometry based assays were established and $CD4^{TNF\text{-}mono}$ T-cell lines were generated. The gained knowledge about this T-cell subpopulation in the context of HBV infection might be useful to design and establish new immunotherapies against chronic hepatitis B.

# 2 Material

## 2.1 Media

### 2.1.1 Media for cell culture

| Name | Ingredients |
|---|---|
| RPMI-10 | RPMI 1640 medium (Life Technologies GmbH, Gibco; Darmstadt, Germany) supplemented with 10% heat inactivated FCS, 1% penicillin/ streptomycin |
| RPMI-rich | RPMI 1640 medium (Life Technologies GmbH, Gibco; Darmstadt, Germany) supplemented with 10% heat inactivated FCS, 1 mM sodium pyruvate, 1% penicillin/streptomycin and 1% MEM non-essential amino acids (NEAA) |
| T-cell medium | RPMI 1640 medium (Life Technologies GmbH, Gibco; Darmstadt, Germany) supplemented with 10% heat inactivated HS, 1% penicillin/ streptomycin, 1% Hepes, 1% L-Glutamin, and 50 µM β-Mercaptoethanol |

### 2.1.2 Additives for cell culture media

| Name | Manufacturer |
|---|---|
| Fetal calf serum (FCS), heat-inactivated | Life Technologies GmbH, Gibco (Darmstadt, Germany) |
| Human serum (HS), heat-inactivated | In-house production |
| MEM non-essential amino acids (NEAA) | Life Technologies GmbH, Gibco (Darmstadt, Germany) |
| Penicillin/streptomycin | PAA Laboratories GmbH (Cölbe, Germany) |
| Sodium pyruvate | Life Technologies GmbH, Gibco (Darmstadt, Germany) |

## 2.2 Antibodies

### 2.2.1 Stimulating Antibodies

| Antigen | Description (clone) | Concentration (µg/ml) | Manufacturer |
|---|---|---|---|
| CD28 | Monoclonal mouse antibody (L293), binds human CD28 | 1 | B&D, Becton Dickinson (Heidelberg, Germany) |
| CD49d | Monoclonal mouse antibody (L25), binds human CD49d | 1 | B&D, Becton Dickinson (Heidelberg, Germany) |
| CD3 | Monoclonal mouse antibody (OKT3), binds human CD3 | 0.03 | eBioscience (Frankfurt am Main, Germany) |

### 2.2.2 Fluorochrome-labeled antibodies

| Name (clone) | Concentration (µg/ml) | Manufacturer |
|---|---|---|
| Anti-human CD154-FITC (TRAP-1) | 0.5 | B&D, Becton Dickinson (Heidelberg, Germany) |
| Anti-human CD8-FITC (SK1) | 0.31 | B&D, Becton Dickinson (Heidelberg, Germany) |
| Anti-human CTLA4-FITC (A3.4H2.H12) | 12.5 | Biozol, (Eching, Germany) |
| Anti-human TNF-α-FITC (MAb11) | 0.05 | eBioscience (Frankfurt am Main, Germany) |
| Anti-human CD127-PE (hIL-7R-M21) | 0.03 | B&D, Becton Dickinson (Heidelberg, Germany) |
| Anti-human CD8-PE (SK1) | 0.08 | B&D, Becton Dickinson (Heidelberg, Germany) |
| Anti-human MIP1-β-PE (D21-1351) | 0.8 | B&D, Becton Dickinson (Heidelberg, Germany) |
| Anti-human CD4-ECD (SFCI12T4D11) | 0.02 | Beckman Coulter (Krefeld, Germany) |
| Anti-human CD8-ECD (2ST8.5H7) | 0.16 | Beckman Coulter (Krefeld, Germany) |
| Anti-human CD4-PerCP (SK3) | 0.24 | B&D, Becton Dickinson (Heidelberg, Germany) |
| Anti-human CD8-PerCP (SK1) | 0.39 | B&D, Becton Dickinson (Heidelberg, Germany) |
| Anti-human TNFR2-PerCP (22235) | 2.5 | R&D (Wiesbaden, Germany) |
| Anti-human CD3-PerCP-Cy5.5 (SK7) | 0.19 | B&D, Becton Dickinson (Heidelberg, Germany) |
| Anti-human PD1-PerCP-eFluor(eF)710 (J105) | 1.88 | eBioscience (Frankfurt am Main, Germany) |
| Anti-human CD25-PE-Cy7 (BC96) | 3.13 | eBioscience (Frankfurt am Main, Germany) |
| Anti-human CD3-PEC-Cy7 (SK7) | 0.08 | B&D, Becton Dickinson (Heidelberg, Germany) |
| Anti-human CD45RA-PE-Cy7 (L48) | 0.2 | B&D, Becton Dickinson (Heidelberg, Germany) |
| Anti-human CD3-V500 (SP34-2) | 2 | B&D, Becton Dickinson (Heidelberg, Germany) |
| Anti-human CD4-APC (OKT4) | 0.3 | eBioscience (Frankfurt am Main, Germany) |
| Anti-human CD8-APC (SK1) | 0.31 | B&D, Becton Dickinson (Heidelberg, Germany) |
| Anti-human IL-2-APC (5344.111) | 20 | B&D, Becton Dickinson (Heidelberg, Germany) |
| Anti-human Ox40-APC (ACT35) | 3.13 | Life Technologies GmbH, Molecular Probes (Darmstadt, Germany) |

| | | |
|---|---|---|
| Anti-human Tim3-APC (F38-2E2) | 0.75 | eBioscience (Frankfurt am Main, Germany) |
| Anti-human FoxP3-PacB (259D) | 3.75 | BioLegend (Fell, Germany) |
| Anti-human CD8-PacB (DK25) | 0.63 | Biozol, (Eching, Germany) |
| Anti-human CD3-eFluor(eF)450 (OKT3) | 1.25 | eBioscience (Frankfurt am Main, Germany) |
| Anti-human TNF-α-eFluor(eF)450 (MAb11) | 0.05 | eBioscience (Frankfurt am Main, Germany) |
| Anti-human 4-1BB-Alexa Fluor(Al)700 (4B4-1) | 6.25 | Biozol, (Eching, Germany) |
| Anti-human CD3—Alexa Fluor(Al)700 (UCHT1) | 1.25 | B&D, Becton Dickinson (Heidelberg, Germany) |
| Anti-human IFN-γ-Alexa Fluor(Al)700 (B27) | 0.4 | B&D, Becton Dickinson (Heidelberg, Germany) |

## 2.3 Reagents

### 2.3.1 Reagent systems (kits)

| **Name** | **Manufacturer** |
|---|---|
| Cytofix/Cytoperm fixation/permeabilization Kit | B&D, Becton Dickinson (Heidelberg, Germany) |
| FOXP3 Fix/Perm Buffer Set | BioLegend (Fell, Germany) |
| LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (NIR) | Life Technologies GmbH, Invitrogen (Darmstadt, Germany) |
| Naïve $CD4^+$ T cell Isolation Kit II, human | Miltenyi Biotec (Gladbach, Germany) |
| TNF-α Secretion Assay - Detection Kit (PE) | Miltenyi Biotec (Gladbach, Germany) |

### 2.3.2 Buffers and Solutions

| **Name** | **Manufacturer** |
|---|---|
| MACS Buffer, 1x PBS + 0.5% FCS + 2mM EDTA | In-house production |
| 1x PBS | Life Technologies GmbH, Gibco (Darmstadt, Germany) |
| Stain Buffer (FACS Buffer) | B&D, Becton Dickinson (Heidelberg, Germany) |
| FACSClean | B&D, Becton Dickinson (Heidelberg, Germany) |
| FACSFlow | B&D, Becton Dickinson (Heidelberg, Germany) |
| FACSRinse | B&D, Becton Dickinson (Heidelberg, Germany) |

### 2.3.3 Chemicals

| Name | Manufacturer |
|---|---|
| Brefeldin A (BFA) | Sigma-Aldrich, Chemie GmbH (Munich, Germany) |
| Cyclosporin A | Sigma-Aldrich, Chemie GmbH (Munich, Germany) |
| Dimethylsulfoxid (DMSO) | Sigma-Aldrich, Chemie GmbH (Munich, Germany) |
| Ethylenediaminetetraacetic acid (EDTA) | Sigma-Aldrich, Chemie GmbH (Munich, Germany) |
| Ionomycin (I) | Sigma-Aldrich, Chemie GmbH (Munich, Germany) |
| Pancoll separating solution | PAN-Biotech GmbH (Aidenbach, Germany) |
| PEGASYS, pegylated IFN-α 2a | Roche (Basel, Suisse) |
| Phorbol-12-myristrate-13-acetate (PMA) | Sigma-Aldrich, Chemie GmbH (Munich, Germany) |
| Recombinant human IL-2 | PeproTech (Hamburg, Germany) |
| Recombinant human IL-18 | Medical & Biological Laboratories Co., Ltd. (Nagoya, Japan) |
| Trypan blue | Life Technologies GmbH, Gibco (Darmstadt, Germany) |
| Tuerk solution | Sigma-Aldrich, Chemie GmbH (Munich, Germany) |
| Trizol | Life Technologies GmbH, Invitrogen (Darmstadt, Germany) |

## 2.4 Antigens

### 2.4.1 Proteins

| Description | Concentration (µg/ml) | Manufacturer |
|---|---|---|
| *Yeast*-derived recombinant HBV surface antigen (HBsAg), genotype D, subtype adw | 5 | Rhein Biotech (Düsseldorf, Germany) |
| *E.coli*-derived recombinant HBV core antigen (HBcAg), genotype D, subtype adw | 5 | Biochemical research center, University of Latvia (Riga, Latvia) |

### 2.4.2 Hepatitis B virus (HBV) peptide pools

| Sequence | Protein |
|---|---|
| ATVELLSFLP | $core_{11-20}$ |
| LPSDFFPSVR | $core_{19-28}$ |
| YVNTNVGLKI | $core_{88-97}$ |
| CLTFGRETVL | $core_{107-116}$ |

| Sequence | Protein |
|---|---|
| EYLVSFGVWI | $core_{117-126}$ |
| VSFGVWIRTP | $core_{120-129}$ |
| ILSTLPETTV | $core_{139-148}$ |
| STLPETTVVR | $core_{141-150}$ |

| | |
|---|---|
| MDIDPYKEFGATVEL | core$_{1-15}$ |
| LSFLPSDFFPSVRDL | core$_{16-30}$ |
| FLPSDFFPSVRDLLD | core$_{18-32}$ |
| RDLLDTASALYREAL | core$_{28-42}$ |
| PHHTALRQAILCWGE | core$_{50-64}$ |
| GRETVLEYLVSFGVW | core$_{111-125}$ |
| EYLVSFGVWIRTPPA | core$_{117-131}$ |
| VSFGVWIRTPPAYRP | core$_{120-134}$ |
| TVVRRRDRGRSPRRR | core$_{147-161}$ |
| PLGFFPDHQL | preS1$_{21-30}$ |
| MQWNSTAFHQT | preS2$_{1-11}$ |
| LQDPRVRGLYLPA | preS2$_{12-24}$ |
| SISARTGDPV | preS2$_{44-53}$ |
| VLQAGFFLL | S$_{14-22}$ |
| FFLLTRILTI | S$_{20-28}$ |
| RILTIPQSLD | S$_{25-34}$ |
| FLGGSPVCLG | S$_{41-50}$ |
| LVLLDYQGML | S$_{95-104}$ |

| | |
|---|---|
| LLDYQGMLPV | S$_{97-106}$ |
| IPIPSSWAFA | S$_{150-159}$ |
| WLSLLVPFVQ | S$_{172-181}$ |
| GLSPTVWLSA | S$_{185-195}$ |
| SIVSPFIPLL | S$_{207-216}$ |
| PLGFFPDHQLDPAFG | preS1$_{21-35}$ |
| WPAANQVGVGAFGPR | preS1$_{52-66}$ |
| MQWNSTAFHQTLQDP | preS2$_{1-15}$ |
| LQDPRVRGLYLPAGG | preS2$_{12-26}$ |
| FFLLTRILTIPQSLD | S$_{19-33}$ |
| TSLNFLGGSPVCLGQ | S$_{37-51}$ |
| QSPTSNHSPTSCPPI | S$_{54-68}$ |
| CTTPAQGNSMFPSCC | S$_{124-138}$ |
| CTKPTDGNCTCIPIP | S$_{139-153}$ |
| WASVRFSWLSLLVPF | S$_{165-179}$ |
| MMWYWGPNLYNILSP | S$_{197-211}$ |
| FIPLLPIFFCLWVYI | S$_{212-226}$ |

Peptides were all produced by P&E (peptides&elephants Potsdam, Germany) with a purity of ≥90%. Single peptides derived from HBV core (HBc), from HBV surface (HBs) and from HBV polymerase protein were pooled, respectively. Stocks of HBc, HBs and HBp peptide pool contained 20 µM of each peptide and were stored at -80°C. Final concentration of each peptide in the assay was 2 µM while DMSO concentration was below 1%.

### 2.4.3 Influenza-, Epstein-Barr-, Cytomegalovirus (FEC) peptide pool

| **Sequence** | **Origin** |
|---|---|
| GILGFVFTL | Influenza (Flu) |
| FMYSDFHFI | Flu |
| ELRSRYWAI | Flu |
| SRYWAIRTE | Flu |
| ASCMGLIY | Flu |
| VSDGGPNLY | Flu |
| CTELKLSDY | Flu |
| ILRGSVAHK | Flu |
| KTGGPIYKR | Flu |
| LPFDKTTVM | Flu |
| SIIPSGPLK | Flu |
| RVLSFIKGTK | Flu |
| CLGGLLTMV | Epstein-Barr-virus (EBV) |
| DYCNVLNKEF | EBV |

| **Sequence** | **Origin** |
|---|---|
| RAKFKQLL | EBV |
| QAKWRLQTL | EBV |
| RRIYDLIEL | EBV |
| YPLHEQHGM | EBV |
| RVRAYTYSK | EBV |
| RLRAEAQVK | EBV |
| IVTDFSVIK | EBV |
| ATIGTAMYK | EBV |
| RPPIFIRRL | EBV |
| EENLLDFVRF | EBV |
| FLRGRAYGL | EBV |
| AVFDRKSDAK | EBV |
| NLVPMVATV | Cytomegalovirus (CMV) |
| IPSINVHHY | CMV |

| | |
|---|---|
| TPRVTGGGAM | CMV |
| SDEEEAIVAYTL | CMV |

| | |
|---|---|
| EFFWDANDIY | CMV |
| EFFWDANDIY | CMV |

Peptide pool was all produced by Center of AIDS Reagents, NIBSC (Blanche Lane, UK) with a purity of ≥70%. Stock of FEC peptide pool contained 31.25 µg/ml of each peptide and was stored at -20°C. Final concentration of each peptide in the assay was 2 µg/ml while DMSO concentration was below 1%. This peptide pool was used as a positive control in parallel to the HBV-derived peptide pools.

## 2.5 Laboratory Equipment

| **Name** | **Manufacturer** |
|---|---|
| Flow cytometer, LSR II / LSR Fortessa Configuration: http://www.helmholtz-muenchen.de/en/viro/research/platforms/immunmonitoring-platform/index.html | B&D, Becton Dickinson, Immuncytometry Systems (San José, USA) |
| Cell sorter, FACSAria II | B&D, Becton Dickinson, Immuncytometry Systems (San José, USA) |
| Freezing container, Mr. Frosty | Nunc (Wiesbaden, Germany) |

## 2.6 Consumables

| **Name** | **Manufacturer** |
|---|---|
| Cell culture flasks (25-175 $cm^2$) | Sarstedt AG & Co (Nümbrecht, Germany) |
| Cell strainer, 100 µm nylon filter | B&D, Becton Dickinson (Heidelberg, Germany) |
| Conical tubes, BD Falcon (15-50 ml) | B&D, Becton Dickinson (Heidelberg, Germany) |
| Cryo-Tubes, 1.8 ml, externally threaded | Nunc (Wiesbaden, Germany) |
| Disposable Hemocytometer, C-Chip | Biochrom AG, (Berlin, Germany) |
| FACS tubes | Bio-Rad (München, Germany) |
| Microtiter 96-well plates, U-bottom | Costar (Kaiserslautern, Germany) |

## 2.7 Software

| Name | Company |
|---|---|
| FACS Diva Software, Version 6.2 | B&D, Becton Dickinson (San José, USA) |
| FlowJo, Version 9.7 | Treestar (Ashland, USA) |
| Pestle, Version 5 | Mario Roederer, NIH (Bethesda, USA) |
| SPICE, Version 5 | Mario Roederer, NIH (Bethesda, USA) |
| GraphPad Prism Software, Version 5 | GraphPad Software (La Jolla, USA) |
| Microsoft Office 2013 | Microsoft Corporation (Redmond, WA/USA) |

## 2.8 Samples

| cohort | HBV infection status | HCV infection status | HIV infection status | mean age (range) | gender | antiviral therapy |
|---|---|---|---|---|---|---|
| naïve | naïve, vaccinated | naïve | naïve | 37 (23-62) | 6 m, 14 f | no |
| RHB | resolved | negative | negative | 43 (30-65) | 6 m, 1 f | no |
| AHB | acute | 11 negative, 1 positive | negative | 44 (24-76) | 9 m, 3 f | 2 NA, 10 no |
| CHB | chronic | negative | negative | 47 (23-72) | 25 m, 16 f | 1 no, 40 NA, 12 PEG-IFN-α add-on |
| HIV/CHB | chronic | negative | positive | 32 (18-50) | 10 m, 7 f | 4 NA, 13 no |
| CHC | negative | chronic | negative | 55 (31-75) | 11 m, 9 f | 10 no, 10 PEG-IFN-α +Ribavirin |
| HIV/CHC | negative | chronic | positive | 45 (25-63) | 2 m, 2 f, 3 not known | 1 NA, 9 no |
| HIV | negative | negative | positive | 48 (34-62) | 7 m, 3 f | 3 NA, 7 no |

A/C/RHB: acute/chronic/resolved hepatitis B; CHC: chronic hepatitis C; m: male; f: female; NA: nucleot(s)ide analogue

This study was approved by the local ethics committee at Technische Universität München, Ludwig-Maximilians-Universität München and Johannes Gutenberg-Universität Mainz. Prior to enrolment, each patient or healthy, HBV/HCV/HIV naïve volunteer signed a written consent to participate in the trial according to the Helsinki Declaration of ethical guidelines.

# 3 Methods

## 3.1 Isolation and cryopreservation of PBMC

Anti-coagulated whole blood (heparin or EDTA) was collected by venipuncture and kept at room temperature until PBMC isolation (within 8 h). PBMC were separated by Ficoll density gradient centrifugation and washed twice (250 g, 10 min, 21°C) with RPMI1640 medium supplemented with 1% penicillin/streptomycin. Living PBMC were counted by microscopy using tuerk solution for live/dead discrimination and erythrocyte lysis. For cryopreservation, cells were frozen in FCS containing 10% DMSO ($1x10^7$ PBMC/ml) using a controlled-grade freezing container at -80°C. After 20 h PBMC were transferred to liquid nitrogen storage until their use.

## 3.2 Thawing and resting of PBMC and T-cell lines

Cryopreserved PBMC and T-cell lines were thawed at 37°C and washed twice with RPMI-10. Living cells were counted by microscopy using trypan blue staining. For standard resting procedure, cells were incubated for 18 h at 37°C in a humidified atmosphere at 5% $CO_2$ at a concentration of $2x10^6$ cells/ml RPMI-10 in a 15 ml Falcon tube. All samples reached at least >90% viability and >50% recovery.

## 3.3 *Ex vivo* stimulation of HBV-specific T cells

For *ex vivo* stimulation $1x10^6$ viable, overnight rested PBMC were distributed in 150 µl RPMI-10 containing costimulatory antibodies (1 µg/ml anti-CD28 and anti-CD49d) in one well of a 96-well polypropylene U-bottom microtiter plate. Cells were stimulated with peptide pools in a concentration of 2 µM for HBV peptide pools and 2 µg/ml for FEC peptide pool (positive control). A mock stimulated sample was run in parallel to define background activity. After 1 h incubation at 37°C in 5% $CO_2$, 10 µg/ml of the secretion blocker BFA in a total volume of 50 µl RPMI-10 was added to the cell suspension without mixing and incubation was carried out for additional 4 h at 37°C in 5% $CO_2$. After restimulation period cells were stored overnight at 4°C until intracellular cytokine staining (ICS) was performed (see 3.12).

## 3.4 10-days *in vitro* expansion of HBV-specific T cells

$3\text{-}5x10^5$ viable, overnight rested PBMC were distributed in one well of a 96-well polypropylene U-bottom microtiter plate in a total volume of 200 µl RPMI-rich supplemented with 1 µg/ml co-stimulating antibodies CD28 and CD49d (day 0). For *in vitro* expansion of HBV- and FEC-specific T cells 2 µM and 2 µg/ml peptide pool was used, respectively. PBMC with medium alone served as a negative control to define background activity. After 4 days at 37°C in 5% $CO_2$, 50 IU/ml recombinant human IL-2 were added to the cells. As necessary starting from day 7, half of medium was replaced by RPMI-rich supplemented with 5 IU

recombinant IL-2. On day 10, 50 µl of cell culture supernatant was discarded and cells were restimulated with 2 µM HBs or HBc or 2 µg/ml FEC peptide pool. Afterwards 10 µg/ml of BFA in a total volume of 50 µl RPMI-rich was added to the cells. Stimulation was performed for 5 h at 37°C in 5% $CO_2$. After restimulation period cells were stored overnight at 4°C until intracellular cytokine staining was performed (see 3.12).

## 3.5 *In vitro* treatment of PBMC with IL-18 or PEG-IFN-α

After thawing of PBMC from healthy, HBV/HCV/HIV naïve donors, $5x10^5$ cells were treated with different concentrations of recombinant human IL-18 (0, 12.5, 25, 50 and 100 ng/ml) or PEG-IFN-α (PEGASYS; 0, 62.5, 125, 250 and 500 IU/ml) in 200 µl RPMI-rich supplemented with 1 µg/ml co-stimulating antibodies CD28 and CD49d. PBMC were cultivated for 3 or 6 days at 37°C in 5% $CO_2$, followed by a 5 h incubation step with 10 µg/ml of BFA in a total volume of 200 µl RPMI-rich and analysis by ICS (see 3.12) on the following day (storage overnight at 4°C).

## 3.6 Cultivation of PBMC with HBsAg

3-$4x10^5$ PBMC from healthy, HBV/HCV/HIV naïve donors were cultivated with 5 or 50 µg/ml recombinant HBsAg in 200 µl RPMI-rich supplemented with 1 µg/ml co-stimulating antibodies CD28 and CD49d and 50 IU/ml IL-2 in one well of a 96-well polypropylene U-bottom microtiter plate at 37°C in 5% $CO_2$. PBMC with medium alone served as a negative control to define background activity. As necessary, half of medium was changed or cells were split during cultivation. At day 11 one part of the cells was provided with $1x10^4$ irradiated B-LCL (50Gy) and $1x10^5$ irradiated PBMC (35Gy) in RPMI-rich supplemented with IL-2 and HBsAg (final concentration 50 IU/ml and 5 or 50 µg/ml, resp.) and further cultivated for 11 days at 37°C in 5% $CO_2$. After another restimulation round, cells were incubated for 5 h with 10 µg/ml of secretion blocker BFA in a total volume of 200 µl RPMI-rich on day 28 followed by ICS (see 3.12) on the following day (storage overnight at 4°C). The second part of the cells was directly incubated with 10 µg/ml of secretion blocker BFA for 5h in a total volume of 200 µl RPMI-rich at day 14 followed by ICS (see 3.12) on day 15 (storage overnight at 4°C).

## 3.7 Analysis of naïve and memory CD4 T cells for TNF-α production

Thawed and overnight rested PBMC were used for isolation of naïve CD4 T cells using naïve $CD4^+$ T cell Isolation Kit II according to manufacturer information. $1x10^6$ viable naïve $CD4^+$ T cells, PBMC not containing naïve $CD4^+$ T cells and complete PBMC were distributed in 150 µl RPMI-10 containing costimulatory antibodies (1 µg/ml anti-CD28 and anti-CD49d) in one well of a 96-well polypropylene U-bottom microtiter plate, respectively. After 1 h incubation at 37°C in 5% $CO_2$, 10 µg/ml of secretion blocker BFA in a total volume of 50 µl RPMI-10 was added to the cells and incubation was carried out for additional 4 h at 37°C in

5% $CO_2$. After restimulation period cells were stored overnight at 4°C until intracellular cytokine staining (ICS) was performed (see 3.12).

## 3.8 Generation of $CD4^{TNF\text{-}mono}$ T-cell lines

PBMC from donors with chronic hepatitis B were thawed and rested overnight. Cells were washed once with T-cell medium, separated through a cell strainer and counted using trypan blue staining. $1x10^6$ viable PBMC were distributed in 150 µl RPMI-10 containing costimulatory antibodies (1 µg/ml anti-CD28 and anti-CD49d) in one well of a 96-well polypropylene U-bottom microtiter plate. After 1 h incubation at 37°C in 5% $CO_2$ cells were harvested and TNF-α secretion assay was performed according to manufacturer information. In addition to secreted TNF-α, surface of cells was labeled with anti CD4-APC and anti CD3-eF450 during this procedure. Finally, cells were resuspended in 600 µl MACS buffer and stored at 4°C in the dark until cell sorting (performed within 6h). 0.5 mg/ml Propidium iodide was added to the cells immediately before sorting and $CD4^+$ TNF-$\alpha^+$ T cells were sorted by FACSAria II cell sorter. PMT voltages were adjusted with the help of live/dead stained cells for all parameters. 500-1000 sorted cells were co-cultured with $1x10^4$ irradiated B-LCL (50Gy) and $1x10^5$ irradiated PBMC (35Gy) in one well of a 96-well polypropylene U-bottom microtiter plate in 200 µl T-cell medium supplemented with 30 ng/ml OKT3. After 1 day at 37°C in 5% $CO_2$, 50 IU/ml recombinant human IL-2 were added to the cells. During cultivation half of medium was changed and cells were split if necessary. After 14 days $1x10^6$ $CD4^{TNF\text{-}mono}$ T cells were incubated for 5 h with 10 µg/ml BFA in a total volume of 200 µl T-cell medium followed by ICS (see 3.12). Remaining cells were provided with new feeder cells ($1x10^4$ irradiated B-LCL (50Gy) and $1x10^5$ irradiated PBMC (35Gy)) in T-cell medium supplemented with 30 ng/ml OKT3. $CD4^{TNF\text{-}mono}$ T-cell lines were further grown in two expansion rounds at 37°C, 5% $CO_2$ until day 41. Afterwards cells were harvested and living cells were counted by microscopy using trypan blue staining for live/dead discrimination. $5x10^6$ viable cells/ml were either frozen in 10% DMSO containing FCS using a controlled-grade freezing device on -80°C and after 20 h transferred to liquid nitrogen storage or directly used for *in vitro* PEG-IFN-α treatment (see 3.11) or ICS analysis as on day 14.

## 3.9 Generation of autologous B-lymphoblastoid cell lines

EBV-transformed B-lymphoblastoid cell lines (B-LCL) were generated by incubating freshly isolated or cryopreserved PBMC ($2x10^6$ cells/ml RPMI-10) 2 h at 37°C in 5% $CO_2$ with supernatant of the EBV-secreting cell line B95-8 ($10^2$-$10^3$ IU/ml). Afterwards, Cyclosporin A (1 µg/ml) was used to inhibit T cell mediated killing of infected B cells and cells were cultured in RPMI-10 for further expansion. Autologous B-LCL were used as antigen presenting cells to restimulate $CD4^{TNF\text{-}mono}$ T-cell lines with HBV antigens *in vitro* (see 3.10).

## 3.10 Stimulation of $CD4^{TNF\text{-}mono}$ T-cell lines with HBV antigens

$5x10^5$ viable, overnight rested $CD4^{TNF\text{-}mono}$ T cells and $5x10^5$ viable autologous B-LCL were distributed in 150 µl T-cell medium containing costimulatory antibodies (1 µg/ml anti-CD28 and anti-CD49d) in one well of a 96-well polypropylene U-bottom microtiter plate. $CD4^{TNF\text{-}mono}$ T cells were either stimulated with 5 µg/ml HBcAg or HBsAg or with 2 µM HBs or HBc peptide pool and as a positive control with PMA+I (0.02 µg/ml + 0.75 µg/ml, resp.) at 37°C in 5% CO2. Protein stimulation was performed 2 h before peptide and unspecific stimulation to enable antigen processing. A mock stimulated sample was run in parallel to define background activity. 1 h after peptide addition 10 µg/ml BFA was added in a total volume of 50 µl T-cell medium without mixing and incubation was carried out for additional 4 h at 37°C in 5% $CO_2$. After stimulation period cells were stored overnight at 4°C until ICS was performed (see 3.12).

## 3.11 PEG-IFN-α treatment of $CD4^{TNF\text{-}mono}$ T-cell line

$6.25x10^5$ $CD4^{TNF\text{-}mono}$ T cells were cultured with $1x10^4$ irradiated B-LCL (50Gy) and $1x10^5$ irradiated PBMC (35Gy) in 200 µl T-cell medium supplemented with PEG-IFN-α in different concentrations (PEGASYS; 0, 125, 250 and 500 IU/ml) in one well of a 96-well polypropylene U-bottom microtiter plate (day 0). At day 1, 50 IU/ml recombinant human IL-2 were added to the cells followed by further cultivation at 37°C in 5% $CO_2$. Half of medium was changed and cells were split if necessary during cultivation. After 14 days $1x10^6$ $CD4^{TNF\text{-}mono}$ T cells were provided with new feeder cells ($1x10^4$ irradiated B-LCL (50Gy) and $1x10^5$ irradiated PBMC (35Gy)) in T-cell medium supplemented again with PEG-IFN-α in the respective concentration. $CD4^{TNF\text{-}mono}$ T-cell lines were further grown for 14 days at 37°C in 5% $CO_2$ and then incubated for 5 h with 10 µg/ml of secretion blocker BFA in a total volume of 200 µl T-cell medium followed by ICS (see 3.12) on the following day (storage overnight at 4°C).

## 3.12 Intracellular cytokine staining

PBMC or T-cell lines were labeled with LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit in a total volume of 50 µl for 30 min on ice in the dark and washed twice with 200 µl FACS buffer. Afterwards, PBMC were fixed and permeabilized for 20 min on ice in the dark using 100 µl/well Cytofix/Cytoperm Kit. After two wash steps with 200 µl/well Perm/Wash solution (BD Cytofix/Cytoperm Kit), PBMC were labeled intracellularly with the antibodies CD154-FITC, MIP1-β-PE, CD8-ECD, CD4-PerCP, CD45RA-PE-Cy7, TNF-α-eF450, CD3-V500, IL-2-APC and IFN-γ-Al700 in a total volume of 80 µl (Perm/Wash solution plus antibody mix) for 30 min on ice in the dark. Cells were washed twice with 200 µl/well Perm/Wash solution and finally, resuspended in 300 µl FACS buffer for acquisition. Cells were stored cold and dark until acquisition.

## 3.13 Staining of exhaustion marker on $CD4^{TNF\text{-}mono}$ T cells

Labeling of PBMC with LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit was performed in a total volume of 50 µl for 30 min on ice in the dark followed by two wash steps with 200 µl FACS buffer. PBMC were labeled with the antibodies CTLA4-FITC, CD4-ECD, PD1-PerCP-eF710, CD3-V500 and Tim3-APC in a total volume of 80 µl (FACS buffer plus antibody mix) for 30 min on ice in the dark. For gating of PD1 a fluorsescence minus one (FMO) sample was performed in parallel for each donor. PBMC were washed twice with 200 µl FACS buffer followed by fixation and permeabilization for 20 min on ice in the dark using 100 µl/well Cytofix/Cytoperm Kit. After two wash steps with 200 µl/well Perm/Wash solution (BD Cytofix/Cytoperm Kit), PBMC were labeled intracellularly with the antibody TNF-α-eF450 in a total volume of 60 µl (Perm/Wash solution plus antibody mix) for 30 min on ice in the dark. Cells were washed twice with 200 µl/well Perm/Wash solution and finally, resuspended in 300 µl FACS buffer for acquisition. Cells were stored cold and dark until acquisition.

## 3.14 Staining of $T_{reg}$ and activation marker on $CD4^{TNF\text{-}mono}$ T cells

PBMC were labeled with the LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit in a total volume of 50 µl for 30 min on ice in the dark and washed twice with 200 µl FACS buffer. Afterwards, PBMC were stained with the antibodies CD127-PE, CD4-ECD, TNFR2-PerCP, CD25-PE-Cy7, CD3-V500, Ox40-APC and CD137-Al700 in a total volume of 80 µl (FACS buffer plus antibody mix) for 30 min on ice in the dark. For gating of TNFR2 and CD25 a fluorsescence minus two (FMT) sample was performed in parallel for each donor. PBMC were washed twice with 200 µl FACS buffer and fixed and permeabilized for 30 min on ice in the dark using 100 µl/well fixation buffer (FoxP3 Fix/Perm Buffer Set). After two wash steps with 200 µl/well Perm/Wash solution (FoxP3 Fix/Perm Buffer Set) PBMC were labeled intracellularly with the antibodies TNF-α-FITC and FoxP3-PacB in a total volume of 60 µl (Perm/Wash solution plus antibody mix) for 30 min on ice in the dark. Cells were washed twice with 200 µl/well Perm/Wash solution and finally, resuspended in 300 µl FACS buffer for acquisition. Cells were stored cold and dark until acquisition.

## 3.15 Flow cytometry data acquisition

Acquisition of samples was performed within 6 h after staining using a LSR2/LSR Fortessa flow cytometer equipped with a 96-well plate reader and FACSDiva Software version 6.2. PMT voltages were adjusted with the help of live/dead stained cells for all parameters. Mean autofluorescence values were set to approximately $10^2$ for all used fluorochrome channels. Analysis was performed on living lymphocytes using the software FlowJo version 9.7.

## 3.16 Gating Strategy and data interpretation for polychromatic ICS

Gating strategy for analysis of *ex vivo* and after 10-days *in vitro* expansion restimulated PBMC is shown in Figure 3.1. Each gate was set in the negative control sample and adjusted to the peptide stimulated samples with consideration of T-cell receptor downregulation. Two independent audits were performed to control the gating. According to the differential expression of CD45RA, CD154, IFN-γ, TNF-α, IL-2 and MIP1-β 62 reacting CD4 and 30 reacting CD8 T-cell subpopulations were defined for *ex vivo* analysis (CD154 expression was not considered for CD8 T-cell responses). In case of *in vitro* expanded, restimulated PBMC CD45RA did not lead to a proper staining and was therefore excluded from analysis resulting in 30 reacting CD4 and 16 reacting CD8 T-cell subpopulations.

After background subtraction using the software Pestle version 5.0, an individual threshold level was calculated for each subpopulation. This is important for adequate data correction since unspecific background decreases with the number of positive functions. The individual threshold level for each T-cell subpopulation is defined as the 90$^{th}$ percentile of the distribution of negative values from all stimulated samples of the study participants. To exclude outliers the calculated threshold level for one subpopulation was only used if the number of negative values exceeded 5% of total values and if these negative values resulted from at least three different study participants otherwise threshold level was set to 0. After threshold application all values lower than the respective individual threshold level were set to 0. Furthermore, a general threshold (assay detection limit; LoD) of 0.005% was applied for total CD4 and CD8 T-cell responses. Frequencies of single IFN-γ, single TNF-α and IFN-γ plus TNF-α secreting CD4 and CD8 T-cells were calculated by summing up all values of subpopulations which were positive for only IFN-γ, only TNF-α and at least IFN-γ or TNF-α, respectively. Total CD4 and CD8 T-cell response was calculated by summing up all values of subpopulations which were positive for at least one of the cytokines IFN-γ, TNF-α or IL-2. Functional composition of responding T cells is shown as pie charts, showing the portion of responding T-cell subpopulations to the total antigen-specific response according to their functionality (mono- to trifunctional) upon stimulation, using SPICE software. Subpopulations with three functions are depicted in blue, bifunctional cells are depicted in green and monofunctional cells in grey. Pie arcs show the cytokines produced by the different subpopulations upon stimulation. Thereby IFN-γ is shown in black, TNF-α is shown in turquoise and IL-2 in magenta.

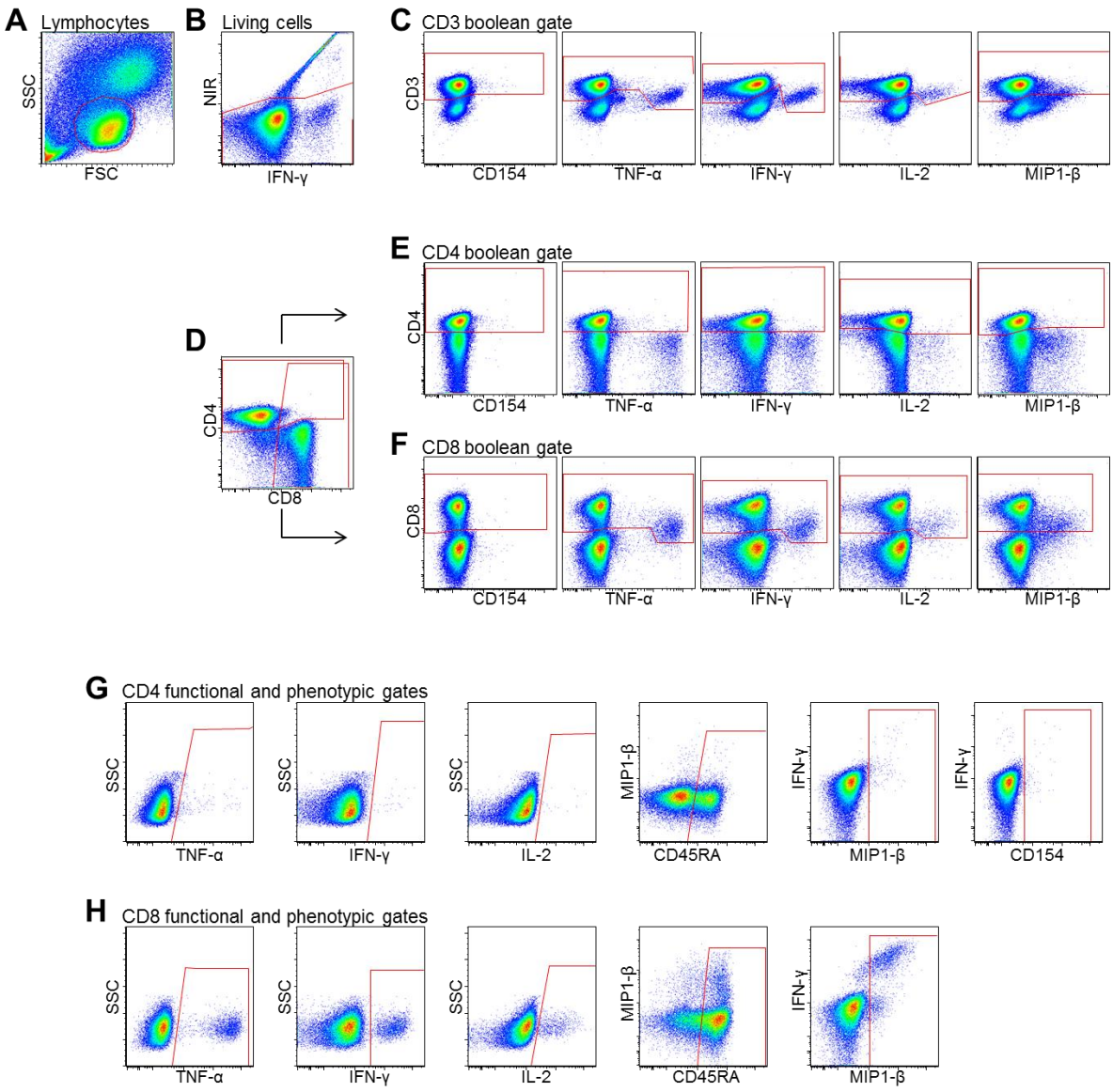



**Figure 3.1: Representative gating strategy for polychromatic intracellular cytokine staining assay.** Lymphocytes were gated based on FSC versus SSC plot (**A**), followed by exclusion of dead cells by NIR staining (**B**). As representatively shown, CD3 cells were gated on all functional markers to account for CD3 downregulation of antigen-specific responding T cells and all these gates were combined with the Boolean operator "OR" to obtain the CD3 cell population (**C**). As representatively shown, CD4 and CD8 cells were gated on all functional markers to account for downregulation of antigen-specific responding T cells and all these gates were combined with the Boolean operator "OR" to obtain the CD4 and CD8 cell population (**E** and **F**, resp.). Thereby CD4 T cells were excluded from the CD8 T-cell population and vice versa (**D**). Once CD4 T-cell population was defined, CD4 T cells (**G**) positive for IFN-γ, TNF-α, IL-2, MIP1-β, CD154 and CD45RA (only in case of *ex vivo* analysis) and CD8 T cells (**H**) positive for IFN-γ, TNF-α, IL-2, MIP1-β and CD45RA (only in case of *ex vivo* analysis) were separately identified by using different plots in which the axis were chosen to provide the best discrimination between positive and negative events. Selection of positive cells for the functional markers was done by comparison with a mock-stimulated sample.

## 3.17 Data interpretation for analysis of exhaustion, activation and $T_{reg}$ marker expression on $CD4^{TNF\text{-}mono}$ T cells

Representative gating strategies for the analyses of exhaustion, activation and Treg marker expression on $CD4^{TNF\text{-}mono}$ T cells are shown in Figure 4.18 and 4.19 (see 4.3.6). Gates for CD25, TNFR2 and PD1 were set in the FMT and FMO sample, respectively whereas the other gates were set in the completely labeled sample. At least two independent audits were performed to control the gating. According to the different expression of the three respective T-cell markers (exhaustion, activation or $T_{reg}$) 8 different subpopulations were defined for $CD4^{TNF\text{-}mono}$ T cells. Composition of markers expressed by $CD4^{TNF\text{-}mono}$ T cells is shown as pie charts, showing the portion of T-cell subpopulations to the total $CD4^{TNF\text{-}mono}$ T cells according to their marker expression, using SPICE. $CD4^{TNF\text{-}mono}$ T-cell subpopulations with three, two, one and no marker are depicted in blue, green, dark grey and light grey, respectively. Pie arcs show the markers expressed by the different $CD4^{TNF\text{-}mono}$ T-cell subpopulations.

## 3.18 Statistical analysis

Nonparametric statistical tests were applied in all cases. Paired Wilcoxon signed rank tests were used to assess significance of change in values between experiment conditions and Mann Whitney tests were used to assess significance of change in values between different groups. To account for multiple comparisons, a closed testing procedure was applied in testing differences of all groups by the Kruskal-Wallis test followed by pairwise comparisons with the Dunn`s multiple comparison test. For correlation analysis the Spearman rank correlation was applied. A confidence interval of 95% was used for all statistical considerations (GraphPad Prism).

# 4 Results

## 4.1 Establishment of patient and control cohorts

Different patient cohorts were established in collaboration with the Departments of Gastroenterology at the University Hospitals in Munich (TUM) and Mainz as well as the Department of Clinical Infectiology and the Division of Infectious Diseases and Tropical Medicine at the University Hospital Munich (LMU). PBMC were isolated from blood by Ficoll density gradient and subsequently cryopreserved. The following samples have been used for this work: (1) HBV/HCV/HIV naïve donors (naïve; n=20), (2) donors with resolved hepatitis B (RHB; n=7), (3) patients with acute hepatitis B (AHB; n=12), (4) patients with chronic hepatitis B (CHB; n=41), (5) patients with chronic hepatitis B and HIV coinfection (HIV/CHB; n=17), (5) patients with chronic hepatitis C (CHC, n=17), (6) patients with chronic hepatitis C and HIV coinfection (HIV/CHC; n=7) and (7) patients with HIV moninfection (HIV; n=10) (see 2.8).

## 4.2 Comparison of HBV-specific T-cell monitoring *ex vivo* and after a 10-days *in vitro* expansion

Manuscript summarizing this part of the results is submitted to Journal of Virology.

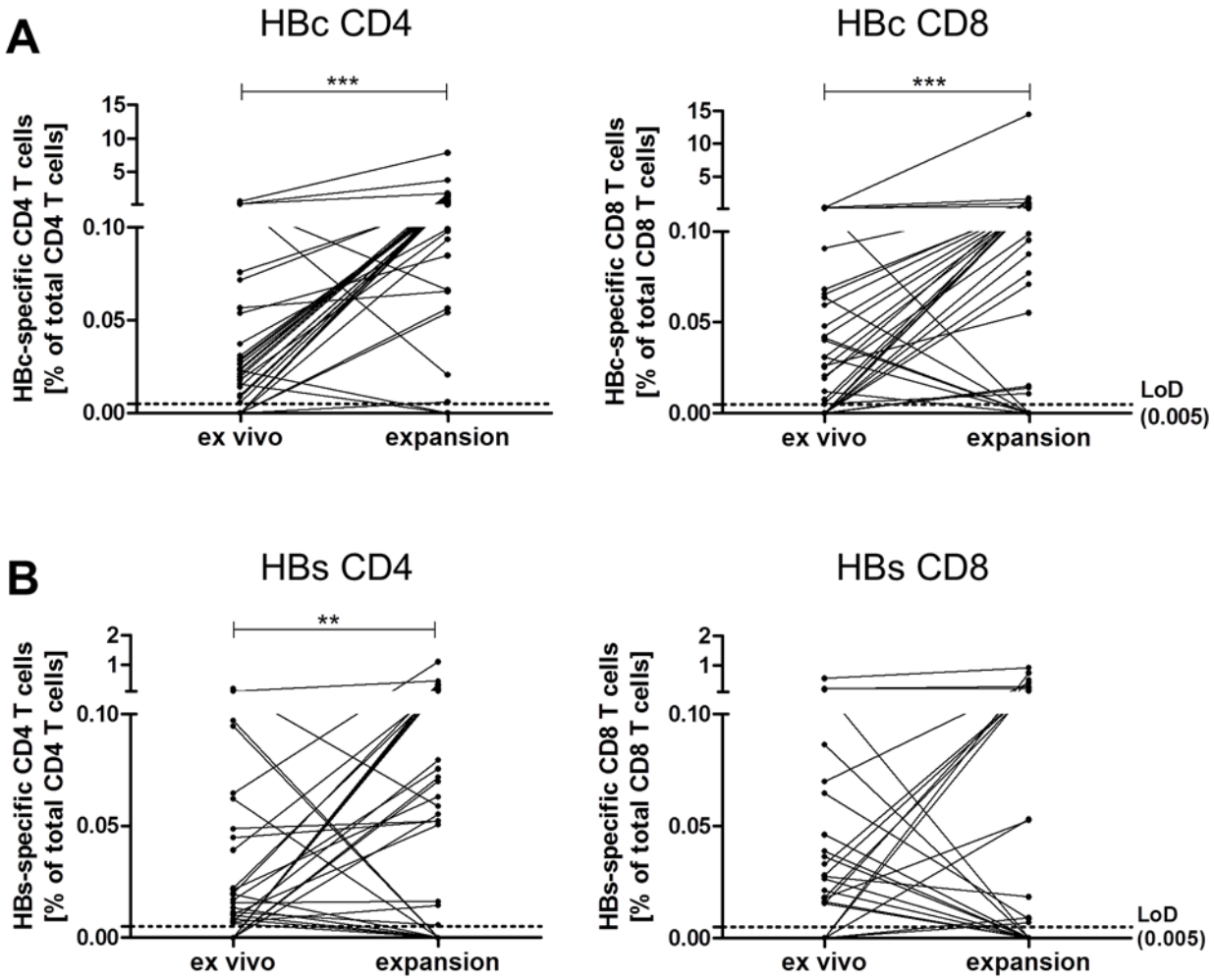
To study HBV-specific T-cell responses two peptide pools including immunodominant T-cell epitopes of HBV core (HBc) and surface (HBs) proteins were used, respectively (see 2.4.2 and [139]). In order to specifically increase low number of peripheral HBV-specific T cells a protocol for a 10-day *in vitro* expansion of PBMC was established. The effect of *in vitro* expansion on quantity and functional profile of HBV-specific T-cell responses compared to *ex vivo* analysis was analysed in 41 donors with different HBV infection status (acute, chronic or resolved infection) using polychromatic ICS (see 3.16).

### 4.2.1 *In vitro* expansion significantly increases numbers of HBV-specific CD4 and CD8 T cells

First the effect of an *in vitro* expansion on quantity of HBV-specific T cells was determined. Analysis of three cytokines (IFN-γ, IL-2, TNF-α) in parallel revealed low median *ex vivo* frequencies of total HBc-specific CD4 and CD8 T cells of 0.02% (range: 0-0.61%) and 0.01% (range: 0-0.3%), respectively (Figure 4.1A). After *in vitro* expansion a significant increase in numbers of total HBc-specific CD4 (Mdn: 0.16%; range: 0-7.8%) and CD8 T cells (Mdn: 0.09%; range: 0-14.46%) was determined. Total HBs-specific CD4 and CD8 T cells were *ex vivo* detectable with median frequencies of 0.01% (range: 0-0.18%) and 0% (range: 0-0.55%), respectively (Figure 4.1B). After *in vitro* expansion a significant increase in numbers of total HBs-specific CD4 T cells (Mdn: 0.05%; range: 0-1.1%) but not of HBs-specific CD8

T cells (Mdn: 0%; range: 0-0.91%) was observed. Overall HBc-specific T cells showed a higher proliferative capacity *in vitro* (median fold increase of HBc-specific CD4 and CD8 T cells: 12.78 and 7.04, resp.) compared to HBs-specific T cells (CD4: 4.26 and CD8: 4.52). However, this was still below the *in vitro* proliferative capacity of Influenza, Epstein-Barr and Cytomegalovirus (FEC)-specific CD4 and CD8 T cells (CD4: 16.69 and CD8: 26.48) which were analysed in parallel in 28 out of 41 donors (Figure 4.1C).

In summary these results show that *in vitro* expansion significantly increases numbers of HBc-specific CD4/CD8 T cells and HBs-specific CD4 T cells but not HBs-specific CD8 T cells.



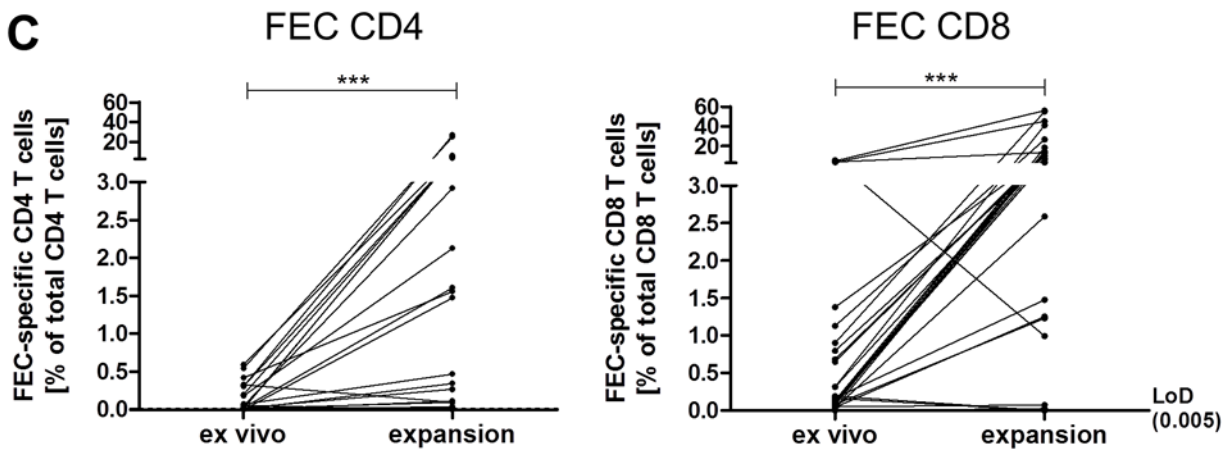



**Figure 4.1: *In vitro* expansion significantly increases numbers of HBV-specific CD4 and CD8 T cells.** CD4 (left panels) and CD8 (right panels) T-cell responses were determined upon restimulation of PBMC *ex vivo* and after 10-days *in vitro* expansion with HBc- (A), HBs- (B) and FEC- (C) derived peptide pools (n=41 and 28 donors for HBs/HBc and FEC, resp.). Total reacting T cells were depicted as frequencies (%) of total CD4 or CD8 T cells, respectively. Background values determined by mock stimulated controls were subtracted and a predefined threshold was applied (see 3.16). (*** $p < 0.001$; ** $p < 0.01$, Wilcoxon matched pairs test); LoD: Limit of detection

### 4.2.2 HBV-specific CD4 and CD8 T-cell numbers after *in vitro* expansion weakly correlate with their precursor frequencies

Next, it was analysed whether the proliferative capacity of HBV-specific T cells *in vitro* correlates with their precursor frequency. Only a weak correlation between numbers of HBc- and HBs-specific CD4 and CD8 T cells detectable *ex vivo* and after expansion was determined (Figure 4.2A and 4.2B, resp.). Proliferative capacity of FEC-specific T cells *in vitro* also correlated only moderately with their precursor frequency (Figure 4.2C). These results show that precursor frequencies of HBV-specific T cells do not allow for conclusion on proliferative capacity *in vitro*.

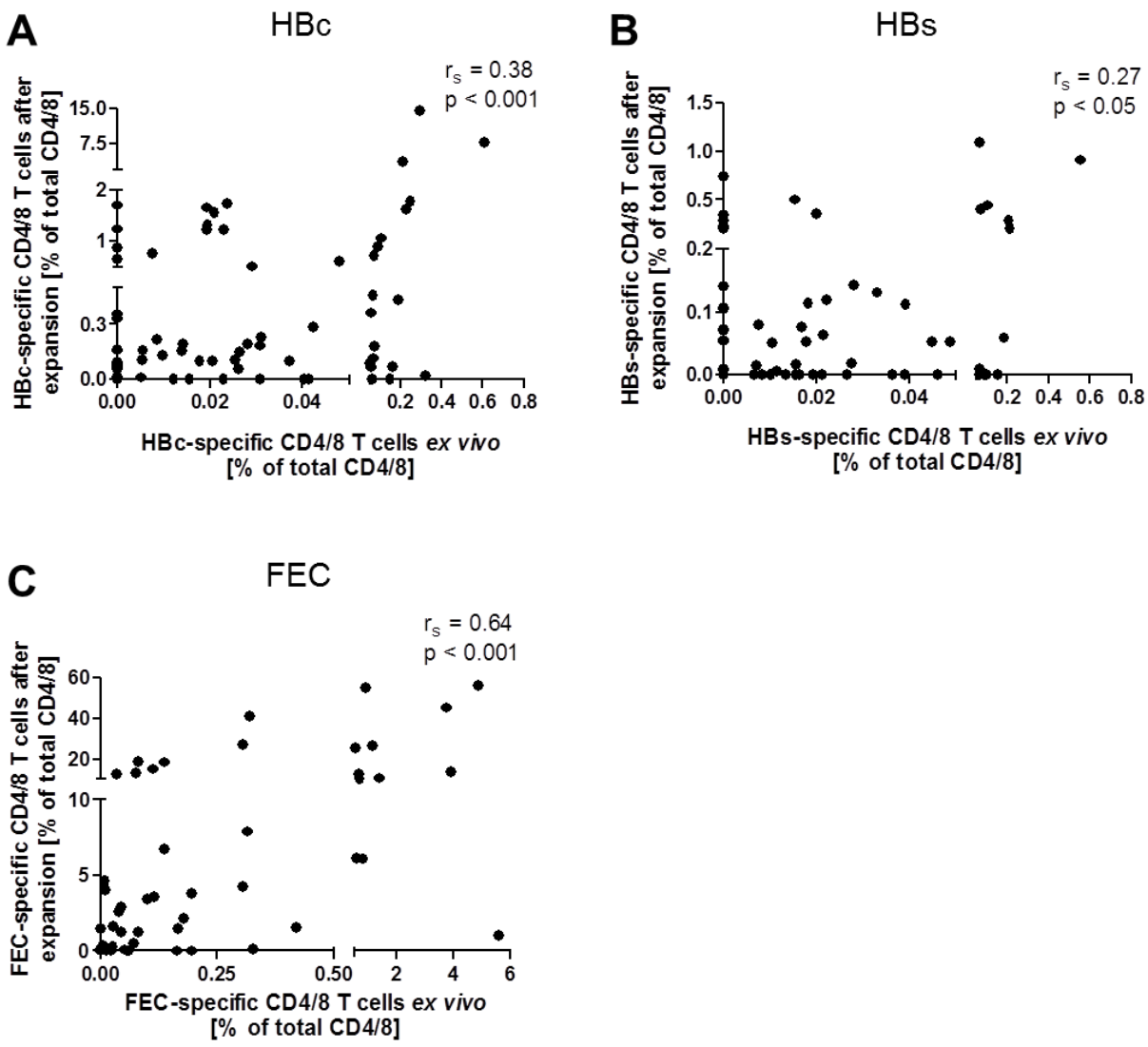



**Figure 4.2: HBV-specific CD4 and CD8 T-cell numbers after *in vitro* expansion weakly correlate with their precursor frequencies.** Correlation of total CD4 and CD8 T-cell responses detectable upon restimulation of PBMC with HBc- (A), HBs- (B) and FEC- (C) derived peptide pools *ex vivo* and after *in vitro* expansion (n=41 and 28 donors for HBs/HBc and FEC, resp.). Background values as determined in mock stimulated controls were subtracted and a predefined threshold was applied before calculating the total response (see 3.16). ($r_S$: Spearman`s rank correlation coefficient)

### 4.2.3 Proliferative capacity of HBV-specific T cells *in vitro* is independent of the donor`s HBV infection status

As in contrast to acute self-limiting infection, chronic hepatitis B is characterized by exhausted HBV-specific T-cell responses, it was analysed whether the donor´s HBV infection status influences the proliferative capacity of HBV-specific T cells *in vitro*. Considering all HBV-specific T-cell responses combined, response rate after expansion was comparable between patients with acute and chronic hepatitis B (Table 4.1). Further fold change of HBV-specific T-cell responses after expansion did not significantly differ between patients with

chronic and acute hepatitis B (Figure 4.3). In summary these data indicate that the capacity of HBV-specific T cells to proliferate *in vitro* is independent of the donor´s HBV infection status.

**Table 4.1: T-cell response rate after expansion according to HBV infection status.**

| reacting T cells | AHB | CHB |
|---|---|---|
| HBc-specific CD4 | 100% (9/9) | 92% (24/26) |
| HBc-specific CD8 | 88% (7/8) | 78% (18/23) |
| HBs-specific CD4 | 63% (5/8) | 78% (18/23) |
| HBs-specific CD8 | 63% (5/8) | 69% (11/16 ) |

A/CHB: acute/chronic hepatitis B

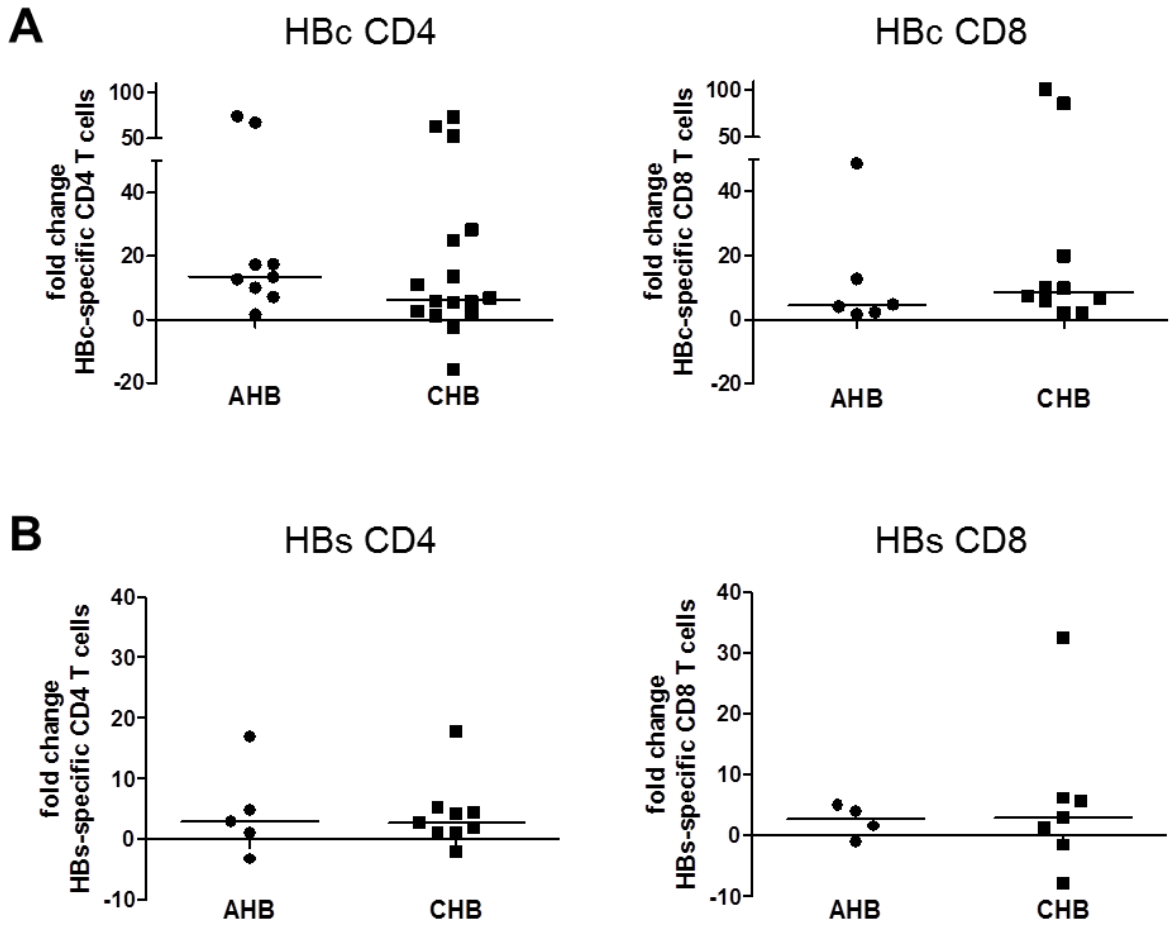



**Figure 4.3: Proliferative capacity of HBV-specific T cells *in vitro* is independent of the donor`s HBV infection status.** Fold change of total CD4 (left panels) and CD8 (right panels) T-cell responses

after restimulation with HBc- (A) and HBs- (B) derived peptide pools *ex vivo* and after *in vitro* expansion. Fold change was defined as ratio of total specific T cells *ex vivo* and after *in vitro* expansion (see Table 4.2 and 4.3). (not significant, Mann Whitney test); A/CHB: acute/chronic hepatitis B

### 4.2.4 Proliferative capacity of HBc-specific CD4 T cells *in vitro* negatively correlates with serum HBsAg and ALT levels in donors with acute hepatitis B

To assess whether the proliferative capacity of HBV-specific T cells *in vitro* is of clinical relevance a potential correlation with serum HBsAg and ALT levels was analysed. In individuals with chronic hepatitis B only a very weak correlation was observed (data not shown). In individuals with acute hepatitis B, however, a strong negative correlation of serum HBsAg as well as ALT levels and the proliferative capacity of HBc-specific CD4 T cells *in vitro* was observed ($r_s$ = -0.9 and $r_s$ = -0.79, resp.; Figure 4.4A and 4.4B, resp.).

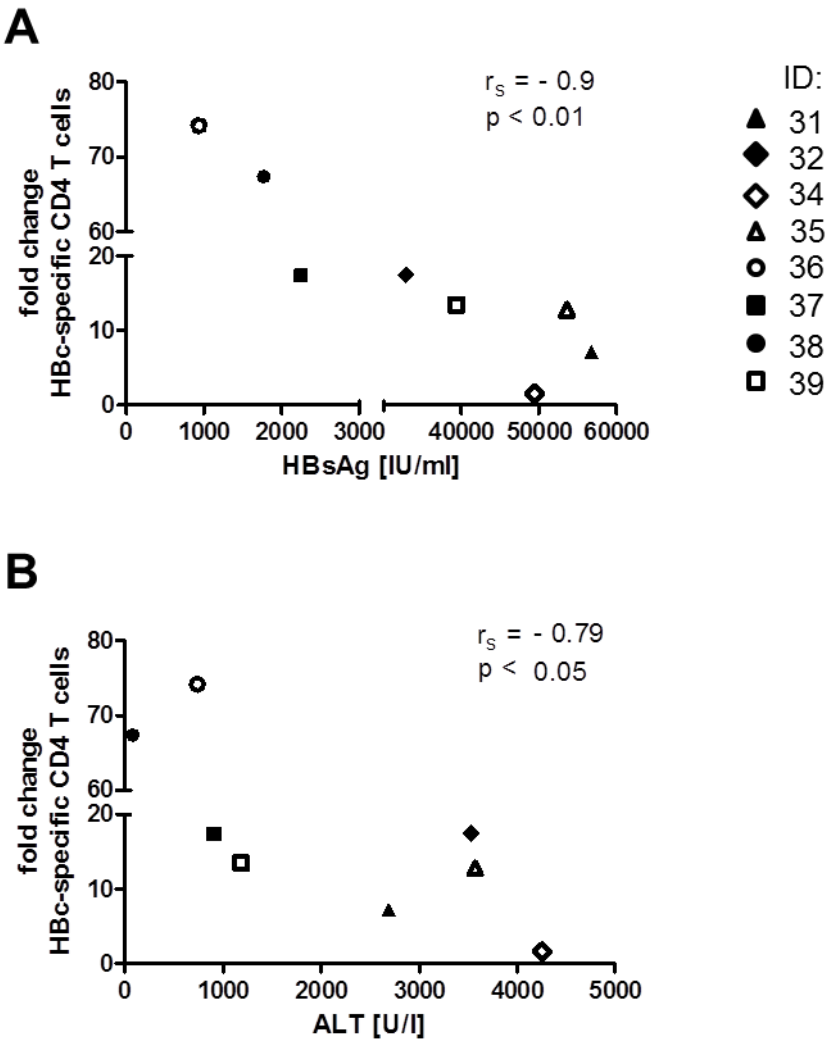


**Figure 4.4: Proliferative capacity of HBc-specific CD4 T cells *in vitro* negatively correlates with serum HBsAg and ALT levels in donors with acute hepatitis B.** Correlation of serum HBsAg (A) and ALT (B) levels with proliferative capacity (fold change) of total HBc-specific CD4 T cells *in vitro* in patients with acute hepatitis B (n=8). Fold change was defined as ratio of total HBc-specific CD4 T cells *ex vivo* and after *in vitro* expansion (see Table 4.2A). Symbols represent different patients. ($r_S$: Spearman`s rank correlation coefficient)

### 4.2.5 *In vitro* expansion increases sensitivity of HBc- but not HBs-specific T-cell monitoring

As *in vitro* expansion of PBMC is commonly used prior to a T-cell monitoring to increase assay sensitivity, HBc- and HBs-specific CD4 and CD8 T-cell response rates *ex vivo* and after *in vitro* expansion were compared (Table 4.2 and 4.3). 61% and 44% of all individuals showed a detectable HBc-specific CD4 and CD8 T-cell response under both assay conditions, respectively (black group Table 4.2A and 4.2B, resp.). However, HBs-specific CD4 and CD8 T-cell responses were detectable in only 37% and 27% of all individuals in both assay setups, respectively (black group Table 4.3A and 4.3B, resp.). In contrast FEC-specific CD4 and CD8 T cells were detectable in 61% and 89% of all individuals *ex vivo* and after expansion, respectively (black group Table 4.4A and 4.4B, resp.). Beside donors showing a detectable T-cell response in both assay setups also donors showing only a detectable T-cell response *ex vivo* or after expansion were observed. Therefore the overall response rate *ex vivo* and after expansion was compared in addition. Response rate of HBc-specific CD4 and CD8 T cells was 22% and 9% higher after expansion compared to *ex vivo* analysis, respectively (Table 4.5). In contrast expansion reduced response rate of HBs-specific CD8 T cells from 80% to 64% while response rate of HBs-specific CD4 T cells was comparable (72% vs. 75%, resp.). Unexpectedly, in about 20% of the donors (CD4: 8/41; CD8: 9/41; grey group Table 4.3A and 4.3B) a HBs-specific T-cell response was detectable *ex vivo* but not after *in vitro* expansion.

*In vitro* expansion of FEC-specific CD4 and CD8 T cells could not further increase assay sensitivity (response rate *ex vivo* and after expansion: CD4: 84% vs. 84%; CD8: 96% vs. 93%, resp.; Table 4.5). In summary the obtained results show that *in vitro* expansion increases sensitivity of HBc- but not HBs-specific T-cell monitoring.

**Table 4.2: Total frequencies of HBc-specific CD4 and CD8 T cells *ex vivo* and after expansion.**

**A** **CD4 HBc**

| ID | HBV infection status | *ex vivo* | expansion | fold change |
|---|---|---|---|---|
| 9 | CHB | 0.324 | 0.021 | -15.79 |
| 29 | CHB | 0.164 | 0.066 | -2.47 |
| 8 | CHB | 0.057 | 0.065 | 1.16 |
| 34 | AHB | 0.054 | 0.085 | 1.58 |
| 27 | CHB | 0.076 | 0.180 | 2.38 |
| 14 | CHB | 0.037 | 0.099 | 2.66 |
| 24 | CHB | 0.018 | 0.098 | 5.52 |
| 1 | CHB | 0.026 | 0.150 | 5.68 |
| 25 | CHB | 0.031 | 0.182 | 5.91 |
| 16 | CHB | 0.028 | 0.192 | 6.81 |
| 31 | AHB | 0.248 | 1.775 | 7.16 |
| 33 | AHB | 0.072 | 0.724 | 10.11 |
| 23 | CHB | 0.014 | 0.155 | 11.05 |
| 35 | AHB | 0.610 | 7.798 | 12.78 |
| 39 | AHB | 0.014 | 0.192 | 13.48 |
| 7 | CHB | 0.010 | 0.132 | 13.60 |
| 37 | AHB | 0.214 | 3.716 | 17.35 |
| 32 | AHB | 0.029 | 0.508 | 17.47 |
| 10 | CHB | 0.009 | 0.214 | 24.94 |
| 2 | CHB | 0.006 | 0.157 | 28.38 |
| 20 | CHB | 0.023 | 1.226 | 53.11 |
| 19 | CHB | 0.019 | 1.219 | 63.15 |
| 38 | AHB | 0.019 | 1.310 | 67.36 |
| 17 | CHB | 0.024 | 1.736 | 73.10 |
| 36 | AHB | 0.021 | 1.551 | 74.19 |
| 26 | CHB | 0.016 | neg. | |
| 11 | CHB | 0.023 | neg. | |
| 12 | CHB | neg. | 0.006 | |
| 40 | RHB | neg. | 0.054 | |
| 13 | CHB | neg. | 0.056 | |
| 21 | CHB | neg. | 0.094 | |
| 18 | CHB | neg. | 0.158 | |
| 3 | CHB | neg. | 0.160 | |
| 6 | CHB | neg. | 0.330 | |
| 28 | CHB | neg. | 0.355 | |
| 41 | RHB | neg. | 0.867 | |
| 30 | CHB | neg. | 1.240 | |
| 4 | CHB | neg. | neg. | |
| 5 | CHB | neg. | neg. | |
| 15 | CHB | neg. | neg. | |
| 22 | CHB | neg. | neg. | |

**B** **CD8 HBc**

| ID | HBV infection status | *ex vivo* | expansion | fold change |
|---|---|---|---|---|
| 37 | AHB | 0.068 | 0.115 | 1.69 |
| 14 | CHB | 0.005 | 0.011 | 2.08 |
| 11 | CHB | 0.026 | 0.055 | 2.11 |
| 31 | AHB | 0.191 | 0.431 | 2.25 |
| 33 | AHB | 0.025 | 0.103 | 4.05 |
| 39 | AHB | 0.021 | 0.098 | 4.78 |
| 8 | CHB | 0.059 | 0.360 | 6.07 |
| 24 | CHB | 0.042 | 0.283 | 6.69 |
| 40 | RHB | 0.066 | 0.458 | 6.99 |
| 41 | RHB | 0.228 | 1.618 | 7.09 |
| 9 | CHB | 0.031 | 0.229 | 7.37 |
| 26 | CHB | 0.108 | 1.059 | 9.82 |
| 4 | CHB | 0.091 | 0.890 | 9.82 |
| 36 | AHB | 0.048 | 0.610 | 12.72 |
| 13 | CHB | 0.005 | 0.107 | 19.89 |
| 35 | AHB | 0.296 | 14.457 | 48.84 |
| 6 | CHB | 0.019 | 1.659 | 86.08 |
| 17 | CHB | 0.008 | 0.762 | 101.02 |
| 32 | AHB | 0.012 | neg. | |
| 3 | CHB | 0.031 | neg. | |
| 1 | CHB | 0.040 | neg. | |
| 27 | CHB | 0.041 | neg. | |
| 28 | CHB | 0.064 | neg. | |
| 29 | CHB | 0.149 | neg. | |
| 7 | CHB | neg. | 0.014 | |
| 12 | CHB | neg. | 0.015 | |
| 38 | AHB | neg. | 0.071 | |
| 22 | CHB | neg. | 0.077 | |
| 19 | CHB | neg. | 0.087 | |
| 10 | CHB | neg. | 0.095 | |
| 20 | CHB | neg. | 0.329 | |
| 30 | CHB | neg. | 0.651 | |
| 16 | CHB | neg. | 1.700 | |
| 2 | CHB | neg. | neg. | |
| 5 | CHB | neg. | neg. | |
| 15 | CHB | neg. | neg. | |
| 18 | CHB | neg. | neg. | |
| 34 | AHB | neg. | neg. | |
| 21 | CHB | neg. | neg. | |
| 23 | CHB | neg. | neg. | |
| 25 | CHB | neg. | neg. | |

Colored bars indicate the different responder groups. R/A/CHB: resolved/acute/chronic hepatitis B

**Table 4.3: Total frequencies of HBs-specific CD4 and CD8 T cells *ex vivo* and after expansion.**

**A** **CD4 HBs**

| ID | HBV infection status | *ex vivo* | expansion | fold change |
|---|---|---|---|---|
| 31 | AHB | 0.183 | 0.059 | -3.13 |
| 27 | CHB | 0.011 | 0.006 | -2.00 |
| 10 | CHB | 0.016 | 0.016 | 1.05 |
| 9 | CHB | 0.049 | 0.052 | 1.07 |
| 39 | AHB | 0.045 | 0.052 | 1.17 |
| 22 | CHB | 0.007 | 0.015 | 2.04 |
| 11 | CHB | 0.039 | 0.112 | 2.87 |
| 32 | AHB | 0.022 | 0.063 | 2.93 |
| 25 | CHB | 0.104 | 0.442 | 4.26 |
| 24 | CHB | 0.017 | 0.075 | 4.46 |
| 37 | AHB | 0.011 | 0.051 | 4.82 |
| 19 | CHB | 0.022 | 0.119 | 5.37 |
| 40 | RHB | 0.008 | 0.079 | 10.50 |
| 38 | AHB | 0.065 | 1.095 | 16.95 |
| 20 | CHB | 0.020 | 0.355 | 17.76 |
| 30 | CHB | 0.007 | neg. | |
| 34 | AHB | 0.008 | neg. | |
| 33 | AHB | 0.010 | neg. | |
| 14 | CHB | 0.013 | neg. | |
| 21 | CHB | 0.019 | neg. | |
| 36 | AHB | 0.062 | neg. | |
| 26 | CHB | 0.095 | neg. | |
| 15 | CHB | 0.097 | neg. | |
| 29 | CHB | neg. | 0.055 | |
| 2 | CHB | neg. | 0.070 | |
| 1 | CHB | neg. | 0.072 | |
| 23 | CHB | neg. | 0.105 | |
| 8 | CHB | neg. | 0.107 | |
| 3 | CHB | neg. | 0.141 | |
| 28 | CHB | neg. | 0.201 | |
| 16 | CHB | neg. | 0.281 | |
| 18 | CHB | neg. | 0.340 | |
| 4 | CHB | neg. | neg. | |
| 5 | CHB | neg. | neg. | |
| 6 | CHB | neg. | neg. | |
| 7 | CHB | neg. | neg. | |
| 12 | CHB | neg. | neg. | |
| 13 | CHB | neg. | neg. | |
| 17 | CHB | neg. | neg. | |
| 41 | RHB | neg. | neg. | |
| 35 | AHB | neg. | neg. | |

**B** **CD8 HBs**

| ID | HBV infection status | *ex vivo* | expansion | fold change |
|---|---|---|---|---|
| 26 | CHB | 0.065 | 0.008 | -7.72 |
| 7 | CHB | 0.028 | 0.018 | -1.50 |
| 36 | AHB | 0.210 | 0.203 | -1.04 |
| 15 | CHB | 0.205 | 0.288 | 1.40 |
| 31 | AHB | 0.554 | 0.912 | 1.65 |
| 8 | CHB | 0.018 | 0.053 | 2.97 |
| 38 | AHB | 0.033 | 0.131 | 3.97 |
| 33 | AHB | 0.028 | 0.142 | 5.08 |
| 3 | CHB | 0.070 | 0.405 | 5.79 |
| 11 | CHB | 0.018 | 0.113 | 6.24 |
| 9 | CHB | 0.015 | 0.501 | 32.53 |
| 37 | AHB | 0.015 | neg. | |
| 16 | CHB | 0.016 | neg. | |
| 32 | AHB | 0.021 | neg. | |
| 40 | RHB | 0.027 | neg. | |
| 4 | CHB | 0.036 | neg. | |
| 27 | CHB | 0.039 | neg. | |
| 19 | CHB | 0.046 | neg. | |
| 39 | AHB | 0.086 | neg. | |
| 20 | CHB | 0.151 | neg. | |
| 6 | CHB | neg. | 0.007 | |
| 24 | CHB | neg. | 0.009 | |
| 23 | CHB | neg. | 0.053 | |
| 18 | CHB | neg. | 0.229 | |
| 34 | AHB | neg. | 0.739 | |
| 1 | CHB | neg. | neg. | |
| 2 | CHB | neg. | neg. | |
| 5 | CHB | neg. | neg. | |
| 10 | CHB | neg. | neg. | |
| 12 | CHB | neg. | neg. | |
| 13 | CHB | neg. | neg. | |
| 14 | CHB | neg. | neg. | |
| 17 | CHB | neg. | neg. | |
| 41 | RHB | neg. | neg. | |
| 35 | AHB | neg. | neg. | |
| 21 | CHB | neg. | neg. | |
| 22 | CHB | neg. | neg. | |
| 25 | CHB | neg. | neg. | |
| 28 | CHB | neg. | neg. | |
| 29 | CHB | neg. | neg. | |
| 30 | CHB | neg. | neg. | |

Colored bars indicate the different responder groups. R/A/CHB: resolved/acute/chronic hepatitis B

**Table 4.4: Total frequencies of FEC-specific CD4 and CD8 T cells *ex vivo* and after expansion.**

**A** **CD4 FEC**

| ID | HBV infection status | *ex vivo* | expansion | fold change |
|---|---|---|---|---|
| 38 | AHB | 0.326 | 0.094 | -3.46 |
| 31 | AHB | 0.012 | 0.030 | 2.48 |
| 4 | CHB | 0.420 | 1.560 | 3.71 |
| 12 | CHB | 0.015 | 0.092 | 6.32 |
| 9 | CHB | 0.072 | 0.467 | 6.49 |
| 16 | CHB | 0.027 | 0.270 | 10.20 |
| 5 | CHB | 0.590 | 6.118 | 10.37 |
| 1 | CHB | 0.179 | 2.131 | 11.88 |
| 2 | CHB | 0.306 | 4.255 | 13.92 |
| 8 | CHB | 0.195 | 3.794 | 19.46 |
| 10 | CHB | 0.543 | 25.313 | 46.58 |
| 37 | AHB | 0.027 | 1.608 | 59.69 |
| 11 | CHB | 0.005 | 0.342 | 63.51 |
| 14 | CHB | 0.044 | 2.921 | 66.50 |
| 40 | RHB | 0.304 | 26.944 | 88.56 |
| 13 | CHB | 0.012 | 4.050 | 348.51 |
| 18 | CHB | 0.009 | 4.636 | 498.51 |
| 17 | CHB | 0.014 | neg. | |
| 34 | AHB | 0.024 | neg. | |
| 33 | AHB | 0.058 | neg. | |
| 15 | CHB | 0.061 | neg. | |
| 35 | AHB | neg. | 0.028 | |
| 39 | AHB | neg. | 0.038 | |
| 3 | CHB | neg. | 0.112 | |
| 6 | CHB | neg. | 1.473 | |
| 19 | CHB | neg. | neg. | |
| 32 | AHB | neg. | neg. | |
| 41 | RHB | neg. | neg. | |

**B** **CD8 FEC**

| ID | HBV infection status | *ex vivo* | expansion | fold change |
|---|---|---|---|---|
| 16 | CHB | 5.615 | 0.994 | -5.65 |
| 12 | CHB | 0.053 | 0.078 | 1.46 |
| 4 | CHB | 3.927 | 13.620 | 3.47 |
| 38 | AHB | 1.379 | 10.452 | 7.58 |
| 31 | AHB | 0.798 | 6.084 | 7.62 |
| 2 | CHB | 0.166 | 1.478 | 8.89 |
| 37 | AHB | 4.885 | 56.108 | 11.49 |
| 6 | CHB | 3.761 | 45.287 | 12.04 |
| 41 | RHB | 0.685 | 10.077 | 14.71 |
| 32 | AHB | 0.082 | 1.230 | 15.04 |
| 39 | AHB | 0.650 | 12.497 | 19.23 |
| 10 | CHB | 1.130 | 26.750 | 23.67 |
| 40 | RHB | 0.315 | 7.890 | 25.09 |
| 17 | CHB | 0.045 | 1.255 | 27.86 |
| 3 | CHB | 0.116 | 3.580 | 30.83 |
| 5 | CHB | 0.101 | 3.428 | 34.09 |
| 9 | CHB | 0.138 | 6.745 | 48.98 |
| 14 | CHB | 0.905 | 55.120 | 60.89 |
| 34 | AHB | 0.039 | 2.588 | 66.03 |
| 13 | CHB | 0.320 | 41.028 | 128.13 |
| 19 | CHB | 0.138 | 18.342 | 132.69 |
| 15 | CHB | 0.113 | 15.008 | 132.74 |
| 11 | CHB | 0.076 | 13.139 | 173.50 |
| 18 | CHB | 0.081 | 18.514 | 227.19 |
| 33 | AHB | 0.035 | 12.496 | 355.61 |
| 8 | CHB | 0.164 | neg. | |
| 1 | CHB | 0.195 | neg. | |
| 35 | AHB | neg. | 0.027 | |

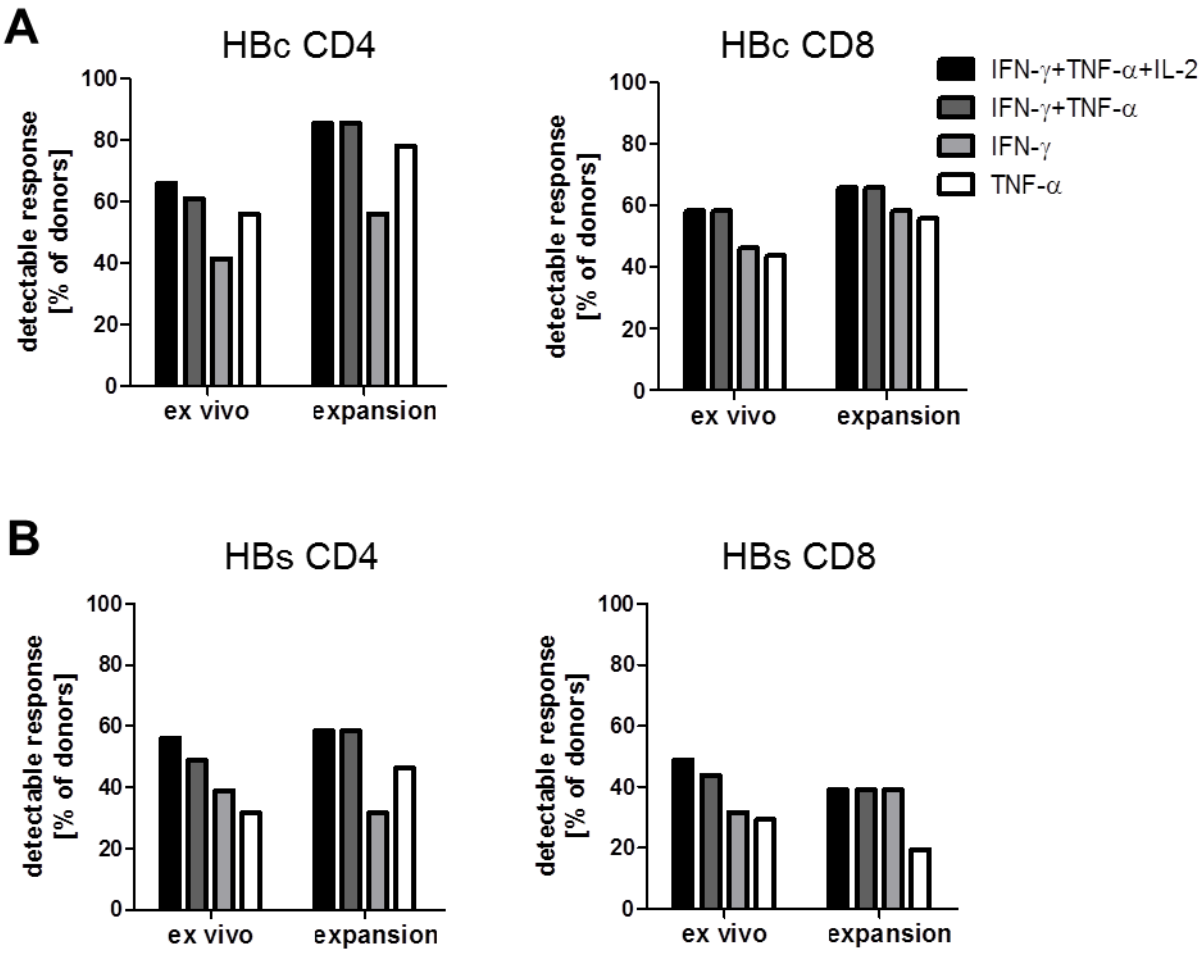
Colored bars indicate the different responder groups. R/A/CHB: resolved/acute/chronic hepatitis B

**Table 4.5**: **T-cell response rate *ex vivo* and after expansion.**

| reacting T cells | *ex vivo* | expansion |
|---|---|---|
| HBc-specific CD4 | 73% (27/37) | 95% (35/37) |
| HBc-specific CD8 | 73% (24/33) | 82% (27/33) |
| HBs-specific CD4 | 72% (23/32) | 75% (24/32) |
| HBs-specific CD8 | 80% (20/25) | 64% (16/25) |
| FEC-specific CD4 | 84% (21/25) | 84% (21/25) |
| FEC-specific CD8 | 96% (26/27) | 93% (25/27) |

### 4.2.6 Multiparametric analysis increases sensitivity of HBV-specific T-cell monitoring

In most studies analysing HBV-specific T-cell responses only IFN-γ or TNF-α production was determined. Therefore the ICS approach analysing IFN-γ, TNF-α and IL-2 in parallel was compared to single cytokine analysis. Single cytokine analysis remarkably reduced the response rate of HBc- and HBs-specific T cells *ex vivo* and after *in vitro* expansion compared to the multiparametric analysis (Figure 4.5A and 4.5B, resp.). Overall TNF-α analysis was more sensitive to detect HBV-specific T cells *ex vivo* and after expansion compared to IFN-γ analysis. Analysis of IL-2 in addition to IFN-γ and TNF-α did not remarkably increase sensitivity of monitoring HBV-specific T cells *ex vivo* and after expansion. In contrast to HBV-specific T cells the single cytokine analysis did not remarkably change the response rate of FEC-specific T cells *ex vivo* and after *in vitro* expansion compared to the multiparametric analysis (Figure 4.5C). Taken together these results show that multiparametric analysis increases sensitivity of HBV-specific T-cell monitoring.



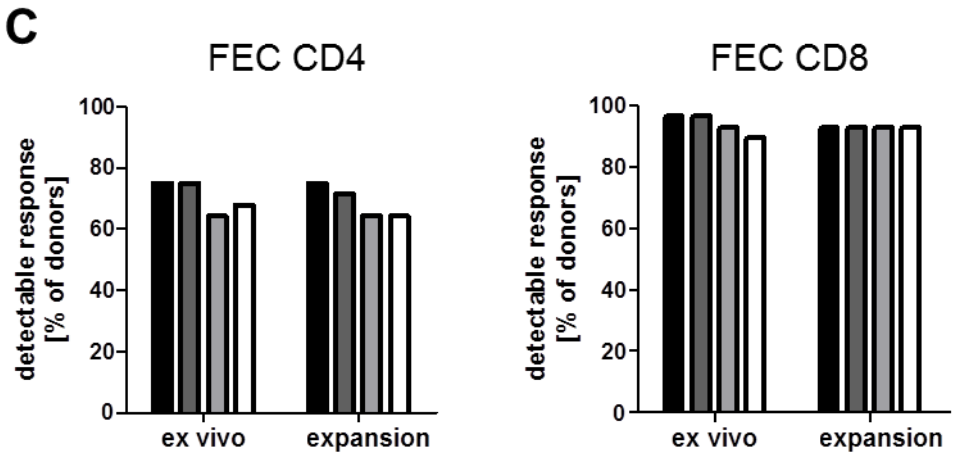



**Figure 4.5: Multiparametric analysis increases sensitivity of HBV-specific T-cell monitoring.** HBc- (A), HBs- (B) and FEC-specific (C) CD4 (left panels) and CD8 (right panels) T-cell responses upon restimulation of PBMC *ex vivo* and after expansion were determined by analysis of IFN-γ, TNF-α and IL-2 (black bars) or IFN-γ and TNF-α in parallel (dark grey bars) as well as IFN-γ (light grey bar) and TNF-α alone (white bar). Background values as determined in mock stimulated controls were subtracted and a predefined threshold was applied before calculating the total response.The number of detectable T-cell responses is depicted as % of the total number of donors.

### 4.2.7 *In vitro* expansion alters functionality of HBV-specific CD4 and CD8 T cells

Finally, the functional profile (i.e. cytokine production) of HBV-specific T cells detectable *ex vivo* and after *in vitro* expansion was compared. *In vitro* expansion resulted in a significant loss of trifunctional HBc-specific CD4 T cells ($p<0.05$) associated with an increase of mono- and bifunctional T cells (Figure 4.6A). Unlike HBc-specific CD4 T cells, numbers of bifunctional HBc-specific CD8 T cells significantly increased after *in vitro* expansion ($p<0.05$) with a simultaneous reduction of monofunctional cells. Trifunctional HBs-specific CD4 T cells were lost after expansion with a simultaneous gain of monofunctional T cells (Figure 4.6B). In contrast trifunctional HBs-specific CD8 T cells increased upon *in vitro* expansion while bifunctional cells were lost. Overall expansion decreased functionality of HBV-specific CD4 T cells whereas it was the opposite for HBV-specific CD8 T cells. Expansion also significantly altered the functional profile of FEC-specific CD4 and CD8 T cells ($p<0.05$ and $p<0.001$, resp.; Figure 4.6C). Overall *in vitro* expanded T cells showed less IL-2 production compared to *ex vivo* analysed T cells, whereas it was the opposite for IFN-γ production of CD8 and TNF-α production of CD4 T cells. Further the degree of functional changes correlated with proliferative capacity. In summary these results show that *in vitro* expansion alters the functionality of HBV-specific CD4 and CD8 T-cell responses with significant impact on the cytokine expression pattern of HBc-specific T cells.

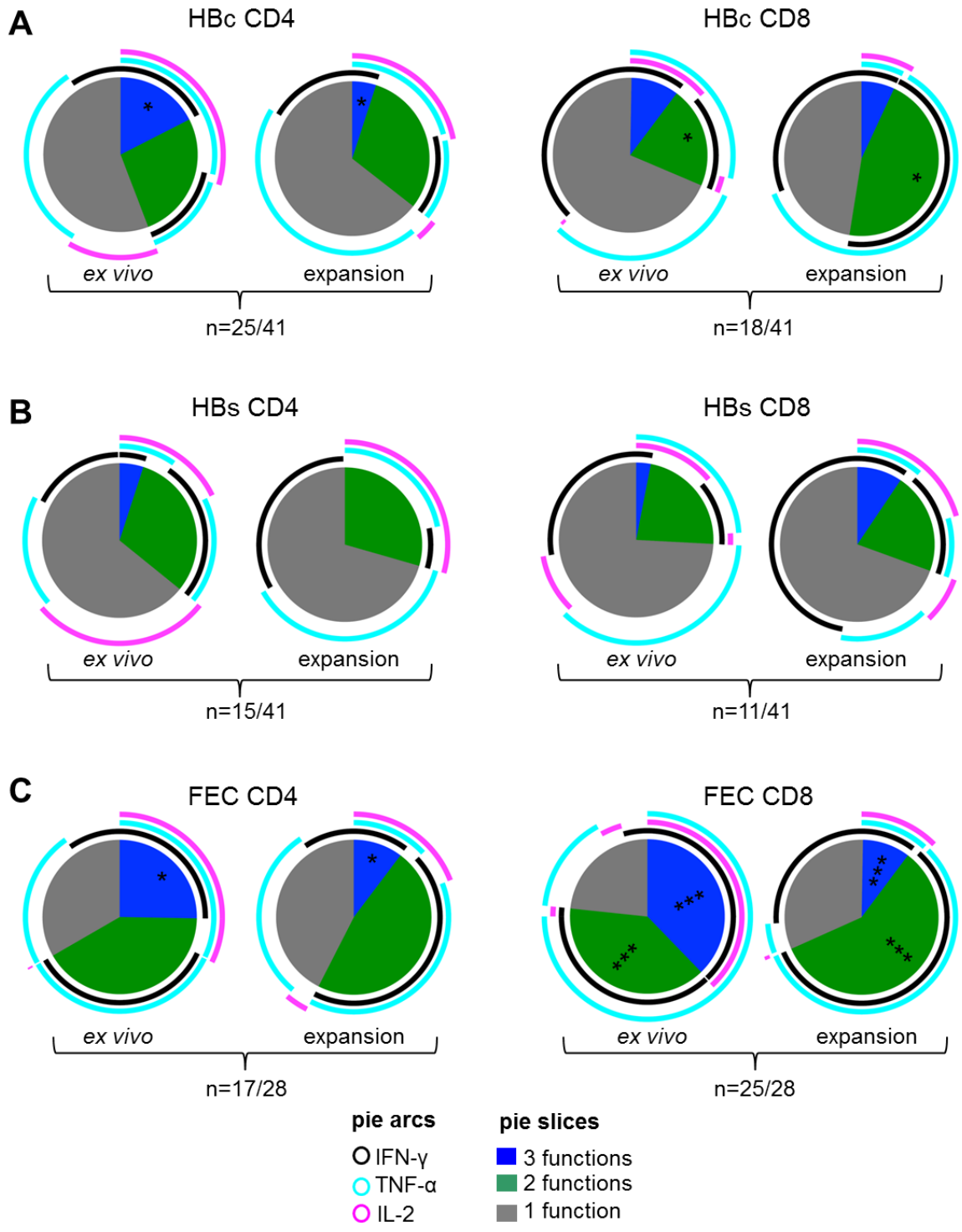



**Figure 4.6: *In vitro* expansion alters functionality of HBV-specific CD4 and CD8 T cells**. Pie charts represent the relative functional composition of total HBc- (A), HBs- (B) and FEC- (C) specific CD4 (left panels) and CD8 (right panels) T-cell responses in all donors with detectable T cells under both assay conditions (number of donors indicated in the figure legend). Each pie slice corresponds to the proportion of T cells positive for a certain combination of functions. Individual marker expression is depicted by the concentric colored arcs surrounding the pie chart. Color codes defining the number (slices) and type (arcs) of T-cell functions are indicated. (*** $p < 0.001$; * $p < 0.05$, Wilcoxon matched pairs test)

### 4.2.8 HBs-specific T cells detectable only *ex vivo* but not after expansion show a predominantly monofunctional phenotype

As mentioned about 20% of the donors showed detectable HBs-specific T-cell responses *ex vivo* but not after expansion (grey group in Table 4.3). Therefore the *ex vivo* cytokine profile of HBs-specific T cells from these donors was compared to donors showing detectable HBs-specific T cells *ex vivo* and >2-fold increased after expansion. HBs-specific T cells detectable only *ex vivo* were predominantly monofunctional compared to HBs-specific T cells detectable >2-fold increased after expansion (Figure 4.7). Further the cells showed less IL-2 production substituted by increased TNF-α (CD4) or IFN-γ (CD8) production compared to HBs-specific T cells detectable >2-fold increased after expansion.

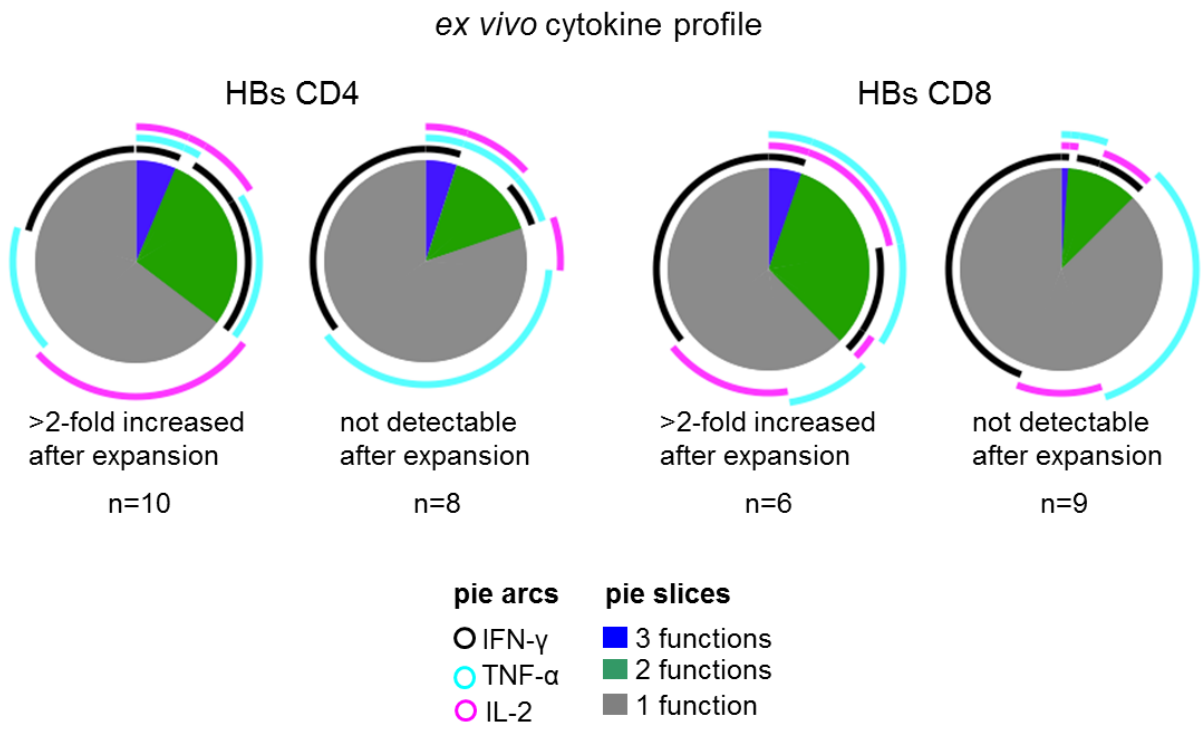



**Figure 4.7: HBs-specific T cells detectable only *ex vivo* but not after expansion show a predominantly monofunctional phenotype.** Pie charts represent the relative functional composition of total HBs-specific CD4 (left panel) and CD8 (right panel) T-cell responses *ex vivo* in donors with detectable T cells *ex vivo* and at least >2-fold increase after expansion and in donors with detectable T cells only *ex vivo* but not after expansion (number of donors is indicated in the figure legend). Each pie slice corresponds to the proportion of T cells positive for a certain combination of functions. Individual marker expression is depicted by the concentric colored arcs surrounding the pie chart. Color codes defining the number (slices) and type (arcs) of T-cell functions are indicated. (not significant, Mann Whitney test)

## 4.3 Phenotypic and functional characterization of CD4$^{TNF\text{-}mono}$ T cells

### 4.3.1 Frequencies of CD4$^{TNF\text{-}mono}$ T cells are elevated in patients with HBV, HCV and HIV infection

During *ex vivo* monitoring of HBV-specific T-cell responses in patients with HBV infection, a yet undescribed monofunctional CD4 T-cell subpopulation producing TNF-α but not IFN-γ, IL-2 or MIP1-β (CD4$^{TNF\text{-}mono}$) in the absence of antigen restimulation *ex vivo* was observed. Significantly lower frequencies of CD4$^{TNF\text{-}mono}$ T cells were determined in healthy, HBV/HCV/HIV naïve individuals (Mdn: 0.15%) and individuals with resolved hepatitis B (Mdn: 0.13%) compared to patients suffering from acute or chronic hepatitis B (Mdn: 0.29% and 0.25%, resp., Figure 4.8). Elevated CD4$^{TNF\text{-}mono}$ T-cell frequencies were also detectable in patients with HCV (Mdn: 0.35%) or HIV (Mdn: 0.52%) monoinfection and HIV/HCV (Mdn: 0.35%) or HIV/HBV (Mdn: 0.46%) coinfection. Taken together these data indicate that infection with HBV, HCV and/or HIV lead to an increased frequency of peripheral CD4$^{TNF\text{-}mono}$ T cells which at least in case of HBV decreases back to levels of HBV/HCV/HIV naïve donors after infection is resolved.

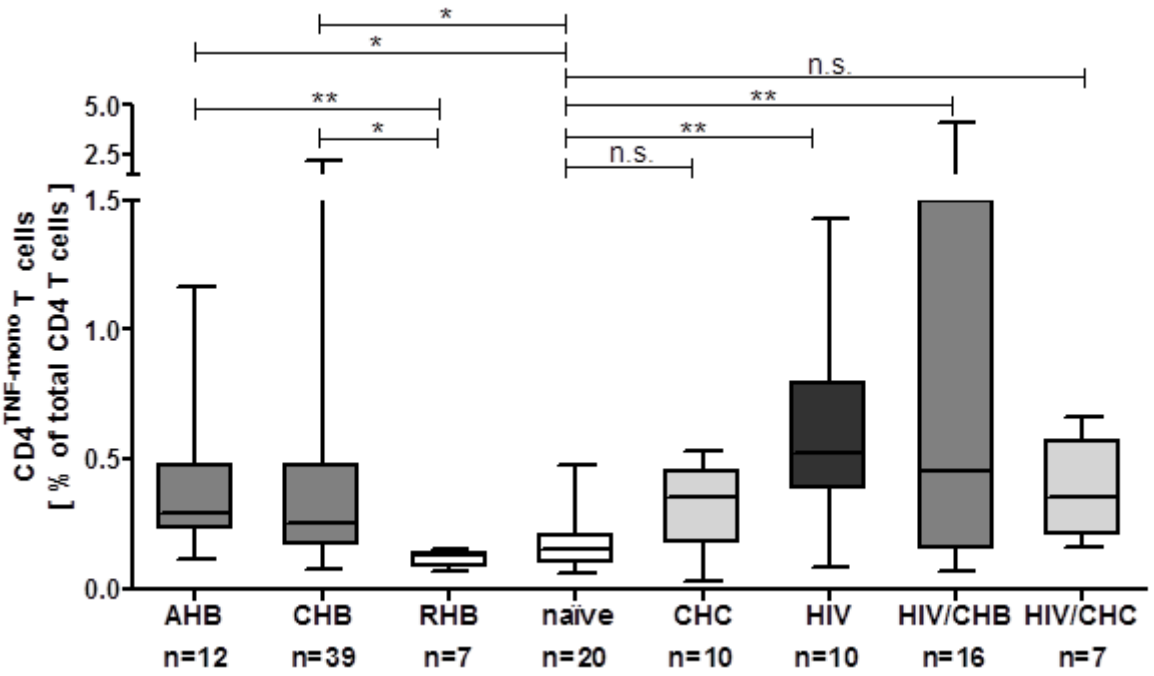



**Figure 4.8: Frequencies of CD4$^{TNF\text{-}mono}$ T cells are elevated in patients with HBV, HCV and HIV infection.** Frequencies of CD4$^{TNF\text{-}mono}$ T cells were determined by ICS without restimulation of PBMC *ex vivo*. CD4 T cells expressing TNF-α but not IL-2, IFN-γ or MIP1-β were depicted as frequencies (%) of total CD4 T cells. Viral infections and number of analysed donors are indicated. Different colors define the patient cohorts. Donors received no PEG-IFN-α treatment at timepoint of analysis. (Kruskal-Wallis test: $p < 0.0001$; Dunn`s multiple comparison test: ** $p < 0.01$; * $p < 0.05$); R/A/CHB: resolved/acute/chronic hepatitis B; CHC: chronic hepatitis C; n.s.: not significant

### 4.3.2 PEG-IFN-α treatment increases frequency of $CD4^{TNF\text{-}mono}$ T cells in patients with chronic hepatitis B and C

In a cohort of 12 patients with chronic hepatitis B receiving PEG-IFN-α for 24 weeks in addition to nucleot(s)ide analogue treatment, significantly increasing frequencies $CD4^{TNF\text{-}mono}$ T cells were observed (Figure 4.9A and 4.10) [70]. Frequencies of $CD4^{TNF\text{-}mono}$ T cells reached a peak at week 8 (w8) of add-on treatment ($p<0.01$), followed by declining frequencies until week 24 (w24). However, frequencies of $CD4^{TNF\text{-}mono}$ T cells were still increased at the end of add-on treatment compared prior treatment values (Mdn: 0.69% vs. 0.23%). Significantly increased frequencies of $CD4^{TNF\text{-}mono}$ T cells were also determined in patients with chronic hepatitis C under PEG-IFN-α treatment compared to untreated patients ($p<0.05$; Figure 4.9B). These results indicate that PEG-IFN-α treatment increases the frequency of $CD4^{TNF\text{-}mono}$ T cells in patients with chronic hepatitis B and C.

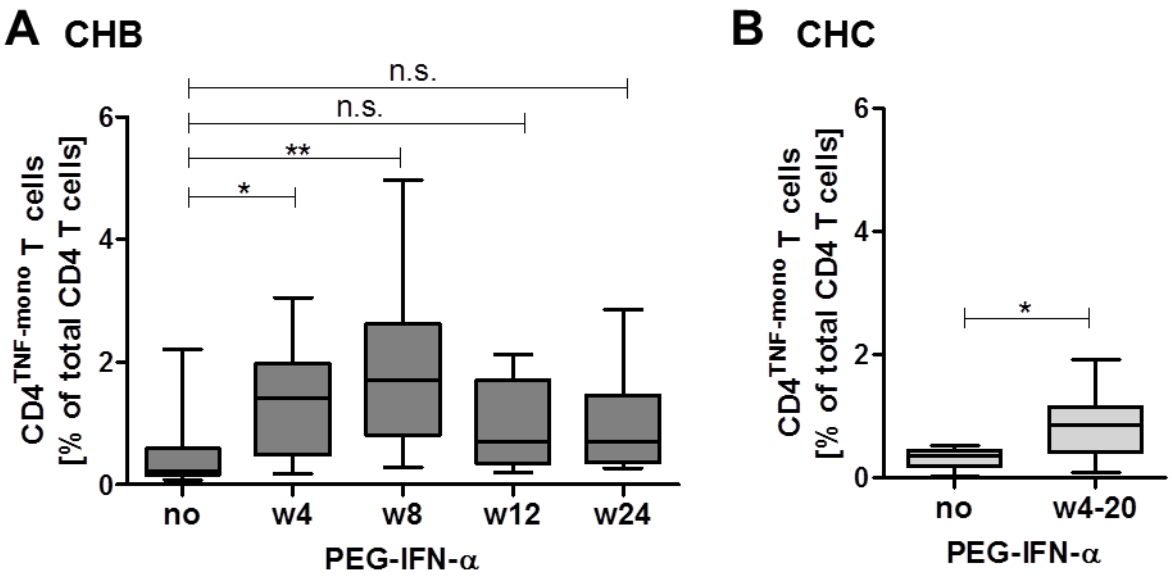



**Figure 4.9: PEG-IFN-α treatment increases frequencies of $CD4^{TNF\text{-}mono}$ T cells in patients with chronic hepatitis B and C.** Frequencies of $CD4^{TNF\text{-}mono}$ T cells were determined by ICS without restimulation of PBMC *ex vivo*. CD4 T cells expressing TNF-α but not IL-2, IFN-γ or MIP1-β were depicted as frequencies (%) of total CD4 T cells. Patients with chronic hepatitis B (n=12; A) and C (n=10; B) with and without PEG-IFN-α treatment were analysed. ((A) Kruskal-Wallis test: $p = 0.0064$; Dunn`s multiple comparison test: ** $p < 0.01$; * $p < 0.05$, (B) Mann Whitney test: * $p < 0.05$); CHB/C: chronic hepatitis B/C; w: treatment week; n.s.: not significant

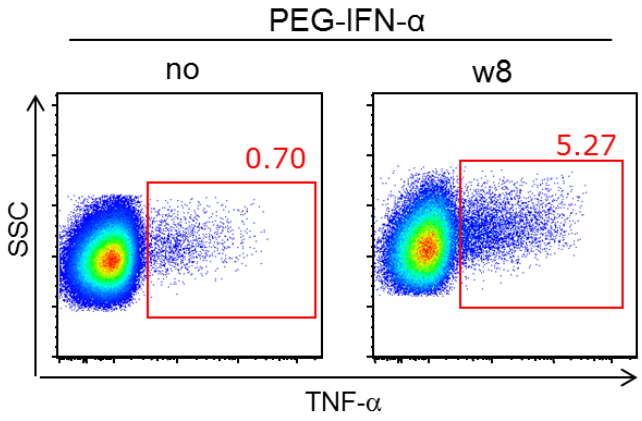



**Figure 4.10: Increased TNF-α production by CD4 T cells induced by PEG-IFN-α treatment in a patient with chronic hepatitis B.** Representative dot plot shows the frequency of CD4 T cells producing TNF-α without restimulation of PBMC *ex vivo* in a patient with chronic hepatitis B without and with 8 weeks (w8) PEG-IFN-α treatment.

### 4.3.3 $CD4^{TNF\text{-}mono}$ T cells produce mainly soluble TNF-α but do not seem to be virus-specific T cells

There are two functional isoforms of TNF-α - soluble and membrane bound - that show different affinities to its receptors TNFR1 and TNFR2 [140]. $CD4^{TNF\text{-}mono}$ T cells from patients with chronic hepatitis B were detectable by ICS if a secretion inhibitor was added (Figure 4.11 left panels). However, this approach does not enable to discriminate between membrane bound and soluble TNF-α. $CD4^{TNF\text{-}mono}$ T cells were not detectable by ICS without addition of a secretion inhibitor and by surface staining of TNF-α leading to the conclusion that $CD4^{TNF\text{-}mono}$ T cells produce mainly soluble TNF-α (Figure 4.11 middle and right panels, resp.).

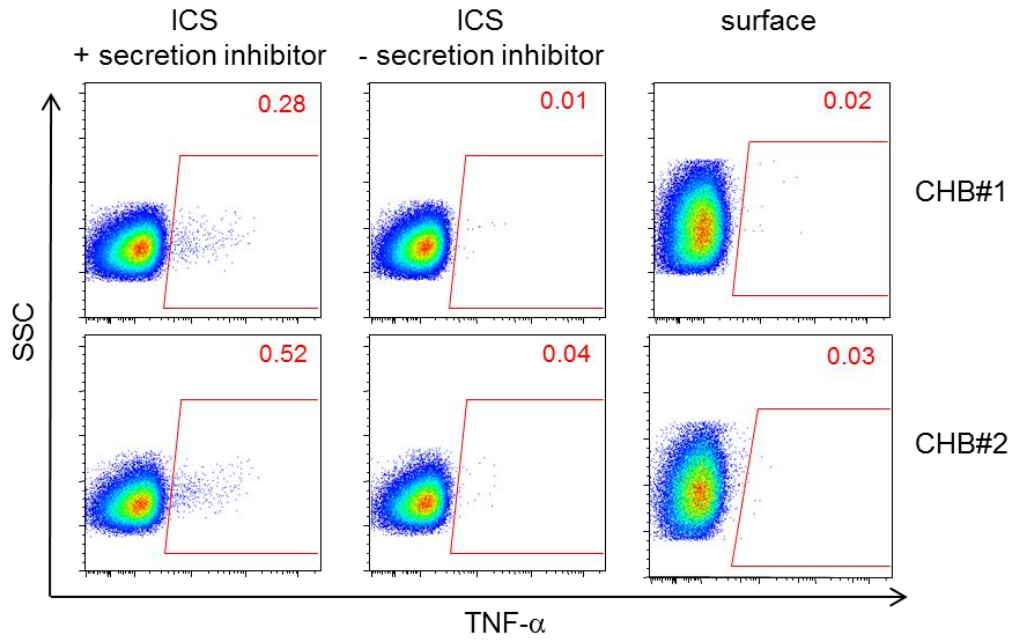



**Figure 4.11: CD4$^{TNF\text{-}mono}$ T cells produce mainly soluble TNF-α.** Isoform of TNF-α produced by CD4 T cells without antigen restimulation *ex vivo* in patients with chronic hepatitis B using flow cytometry. CD4 T cells expressing TNF-α were depicted as frequencies (%) of total CD4 T cells. Results of two representative donors. CHB: chronic hepatitis B

Since elevated CD4$^{TNF\text{-}mono}$ T-cell frequencies were associated with different viral infections it was addressed if these cells are virus-specific T cells. Due to limited patient material and the low frequency of CD4$^{TNF\text{-}mono}$ T cells, CD4$^{TNF\text{-}mono}$ T-cell lines were generated for further characterization. CD4$^{TNF\text{-}mono}$ T cells were sorted *ex vivo* from PBMC of five patients with chronic hepatitis B using a TNF-α secretion assay. The sorted bulk populations of two donors were successfully expanded. Spontaneous TNF-α production by CD4$^{TNF\text{-}mono}$ T-cell lines was lost during *in vitro* culture, since after 14 days only a minority of the cells (line 1: 0.53% and line 2: 0.14%) spontaneously produced TNF-α with a further decline until day 42 (Figure 4.12). However, IL-2 and IFN-γ production remained as expected undetectable during *in vitro* culture. Treatment of a 42-days expanded CD4$^{TNF\text{-}mono}$ T-cell line with PEG-IFN-α (125, 250 and 500 IU/ml) for 4 weeks did not lead to a rescue of TNF-α production (data not shown).

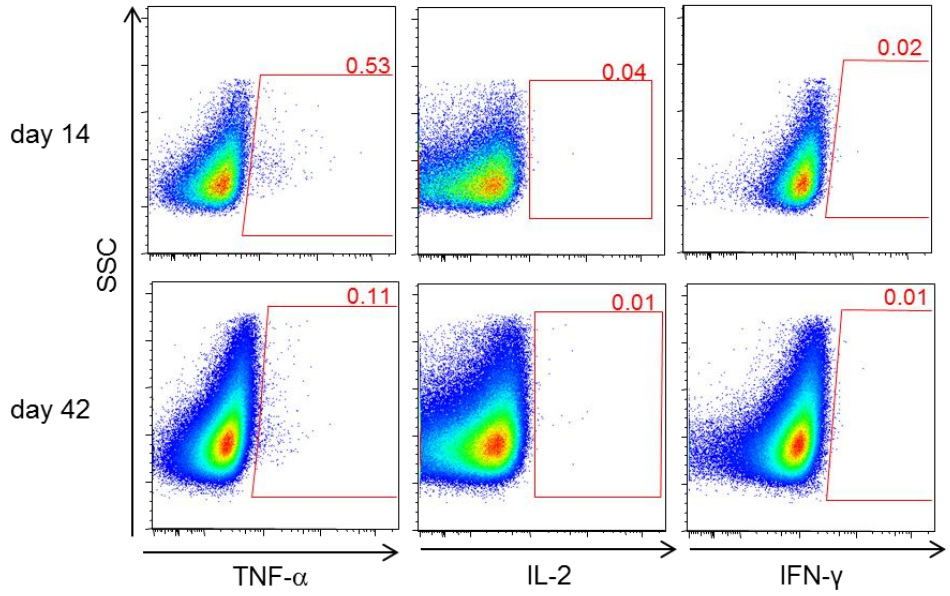



**Figure 4.12: CD4$^{TNF\text{-}mono}$ T cells lose their phenotype during *in vitro* culture.** CD4$^{TNF\text{-}mono}$ T-cell line 1 generated from PBMC of a donor with chronic hepatitis B was analysed for spontaneous TNF-α, IL-2 and IFN-γ production at day 14 and 42 after expansion using ICS.

CD4$^{TNF\text{-}mono}$ T-cell lines were used to analyse virus specificity of CD4$^{TNF\text{-}mono}$ T cells. CD4$^{TNF\text{-}mono}$ T-cell lines from patients with chronic hepatitis B did not produce IL-2, IFN-γ or TNF-α in response to restimulation with HBV antigens (HBc and HBs protein and peptide pool) *in vitro* (Figure 4.13). To test reactivity of CD4$^{TNF\text{-}mono}$ T-cell lines in general the cells were stimulated with PMA+I. Interestingly most of the cells produced TNF-α (52.54%) after unspecific PMA+I stimulation whereas only a minority of the cells produced IL-2 and IFN-γ (5.04% and 7.67%, resp.). In summary, these results show that CD4$^{TNF\text{-}mono}$ T cells from patients with chronic hepatitis B produce mainly soluble TNF-α but do not seem to be virus-specific T cells.

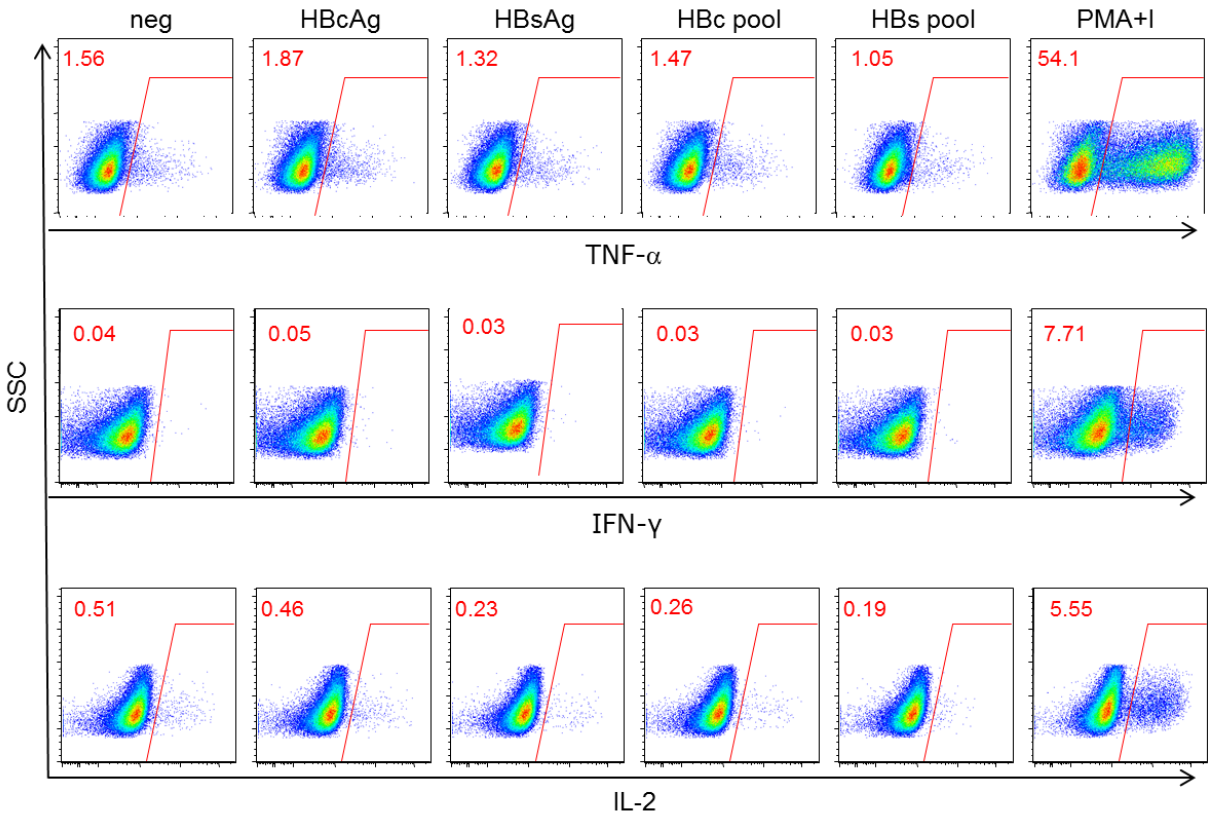



**Figure 4.13: CD4$^{TNF\text{-}mono}$ T-cell lines are not reactive upon restimulation with HBV antigens.** CD4$^{TNF\text{-}mono}$ T-cell line generated from PBMC of a donor with chronic hepatitis B was analysed for TNF-α, IL-2 and IFN-γ production after stimulation with HBc or HBs protein (HBcAg and HBsAg, resp.) or peptide pool (HBc pool and HBs pool, resp.) by flow cytometry. PMA+I was used as a positive control.

### 4.3.4 TNF-α production by CD4 T cells is not triggered by treatment with HBsAg, IL-18 or PEG-IFN-α *in vitro*

As CD4$^{TNF\text{-}mono}$ T cells do not seem to be virus-specific T cells it was addressed how increased frequencies of CD4$^{TNF\text{-}mono}$ T cells are induced in context of viral infections, focusing on chronic HBV infection. Patients with acute and chronic HBV infection typically show high levels of circulating HBsAg. As it is still possible that CD4$^{TNF\text{-}mono}$ T cells were activated by HBsAg *in vivo* although CD4$^{TNF\text{-}mono}$ T-cell lines did not react to HBsAg *in vitro*, PBMC from healthy, HBV/HCV/HIV naïve donors were cultivated over 28 days in presence of high concentrations of HBsAg. No induction of TNF-α production by CD4 T cells was observed in the treated compared to the untreated sample (Figure 4.14A). Next it was analysed if CD4$^{TNF\text{-}mono}$ T cells are IL-18-induced cytokine-producing T cells. Therefore PBMC of healthy, HBV/HCV/HIV naïve donors were treated with different concentrations of recombinant human IL-18 and analysed after 3 and 6 days. No induction of TNF-α production by CD4 T cells was detectable upon IL-18 treatment (Figure 4.14B). Treatment of PBMC from healthy, HBV/HCV/HIV naïve donors with different *in vivo* relevant doses of PEG-IFN-α

did also show no effect on TNF-α production by CD4 T cells after 3 and 6 days of treatment (Figure 4.14C). Taken together these results show that TNF-α production by CD4 T cells is not triggered by HBsAg, IL-18 or PEG-IFN-α treatment *in vitro*.

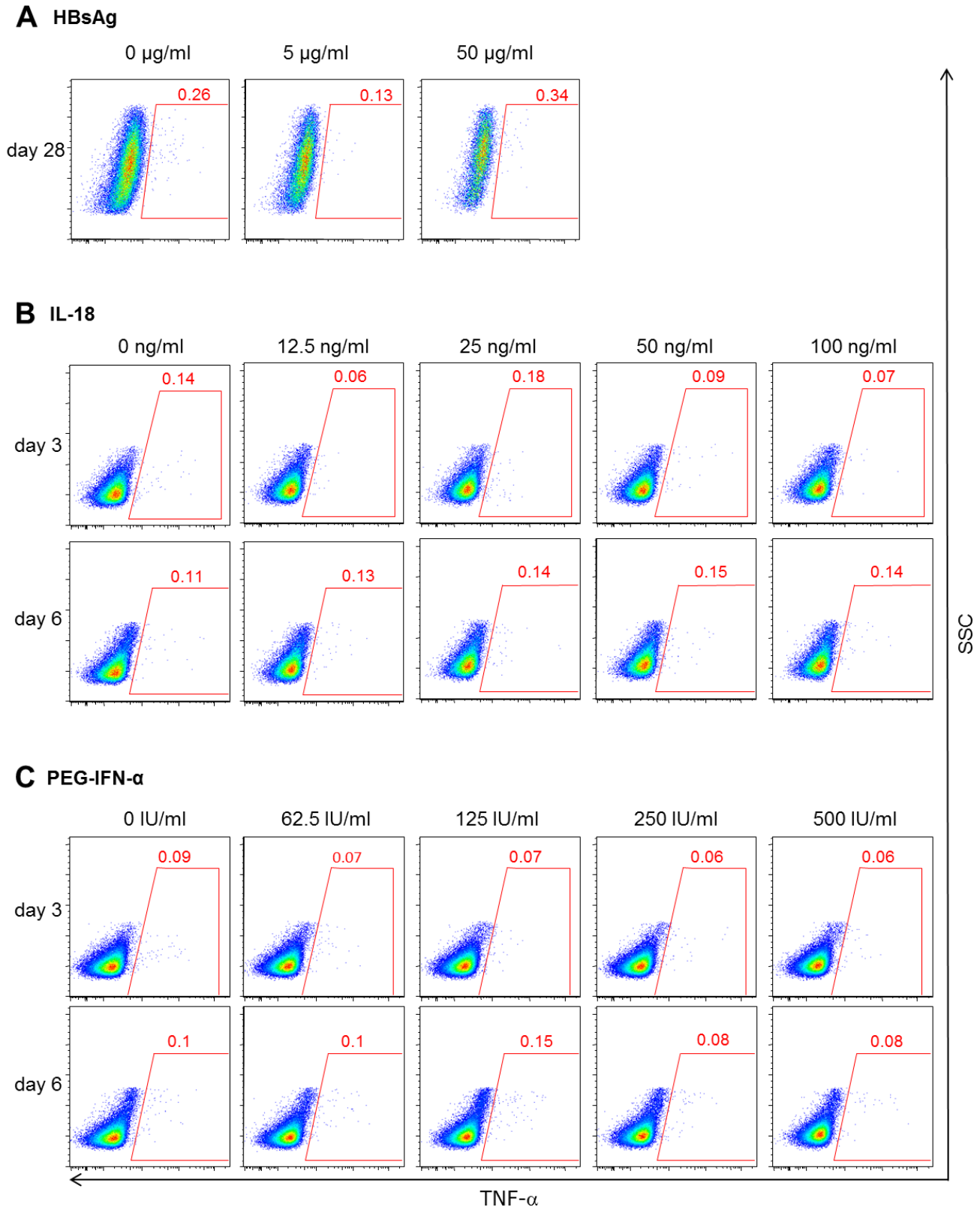



**Figure 4.14: TNF-α production by CD4 T cells is not triggered by treatment with HBsAg, IL-18 or PEG-IFN-α *in vitro*.** PBMC from healthy HBV/HCV/HIV naïve donors were cultivated in the presence of HBsAg (A), IL-18 (B) or PEG-IFN-α (C) as indicated. CD4 T cells were analysed for TNF-α production at the indicated timepoints by ICS.

### 4.3.5 CD4$^{TNF\text{-}mono}$ T cells are predominantly antigen-experienced memory CD4 T cells

Next, the phenotype of CD4$^{TNF\text{-}mono}$ T cells was analysed in patients with chronic hepatitis B. First the maturation status of this CD4 T-cell subpopulation was determined by analysing expression of the naïve T-cell marker CD45RA. In chronically HBV, HCV and HIV infected patients frequencies of CD45RA expressing CD4$^{TNF\text{-}mono}$ T cells were low ranging from 7% (CHC) to 19.49% (CHB) in median (Figure 4.15A). Also in HBV/HCV/HIV naïve donors only a minority of CD4$^{TNF\text{-}mono}$ T cells expressed CD45RA (median frequency 20.43%). Isolation of naïve and memory CD4 T cells confirmed that CD4$^{TNF\text{-}mono}$ T cells mainly represent antigen-experienced memory (CD45RA$^{-}$/CD45RO$^{+}$) CD4 T cells (Figure 4.15B).

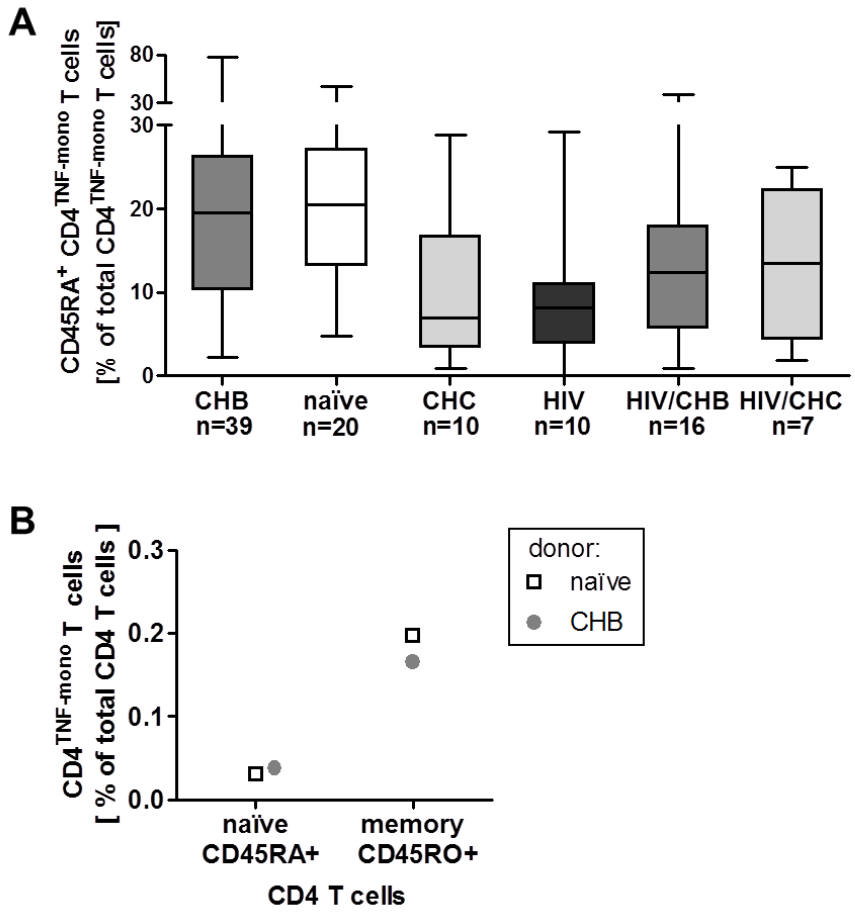



**Figure 4.15: CD4$^{TNF\text{-}mono}$ T cells are predominantly antigen-experienced memory CD4 T cells.** Frequencies of CD4$^{TNF\text{-}mono}$ T cells were determined by ICS without restimulation of PBMC *ex vivo*. CD4$^{TNF\text{-}mono}$ T cells expressing CD45RA were depicted as frequencies (%) of total CD4$^{TNF\text{-}mono}$ T cells (A). CD4$^{TNF\text{-}mono}$ T cells in memory and naïve CD4 T-cell population after MACS sorting of PBMC were depicted as frequencies (%) of total CD4 T cells (B). Donors received no PEG-IFN-α treatment at timepoint of analysis. CHB/C: chronic hepatitis B/C

### 4.3.6 Establishment of multiparametric flow cytometry-based assays for phenotypical characterization of CD4$^{TNF\text{-}mono}$ T cells

To further characterize the phenotype of CD4$^{TNF\text{-}mono}$ T cells two multiparametric flow cytometry-based assays were established to analyse expression of $T_{reg}$, activation and exhaustion marker on CD4$^{TNF\text{-}mono}$ T cells (Figure 4.16 and 4.17, resp.).

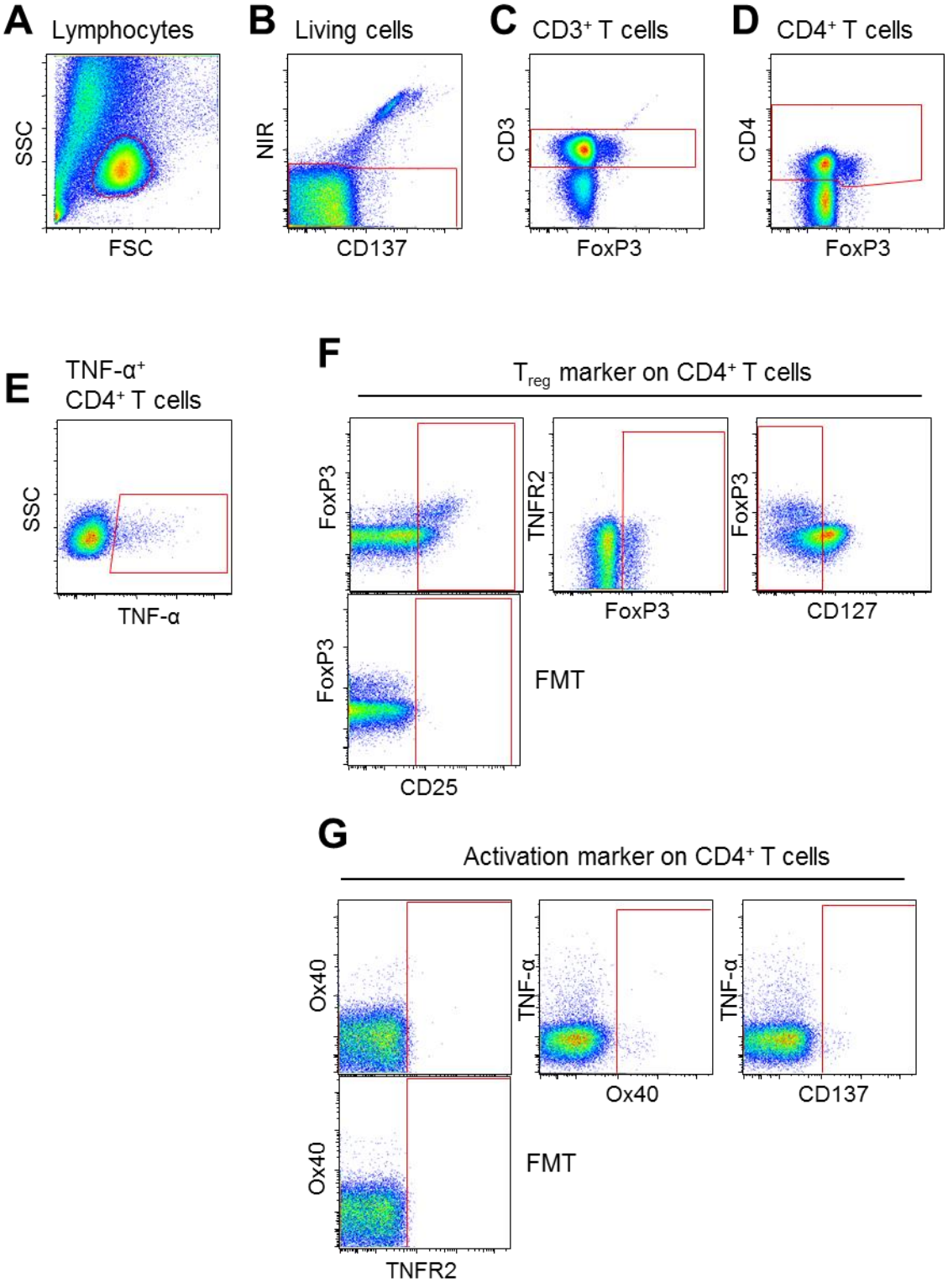


**Figure 4.16: Representative gating strategy for staining of activation and $T_{reg}$ markers on $CD4^{TNF\text{-}mono}$ T cells.** Lymphocytes were gated based on FSC versus SSC plot (**A**), followed by exclusion of dead cells by NIR staining (**B**). CD3 (**C**) and afterwards CD4 cells (**D**) were gated versus FoxP3. Once CD4 T-cell population was defined, CD4 T cells positive for TNF-α (**E**) and for $T_{reg}$ markers CD25, FoxP3 and CD127(low) (**F**) and activation markers TNFR2, Ox40 and CD137 (**G**) were separately identified by using different plots in which the axis were chosen to provide the best discrimination between positive and negative events. CD25 and TNFR2 were gated in the fluorescence minus two (FMT) sample.

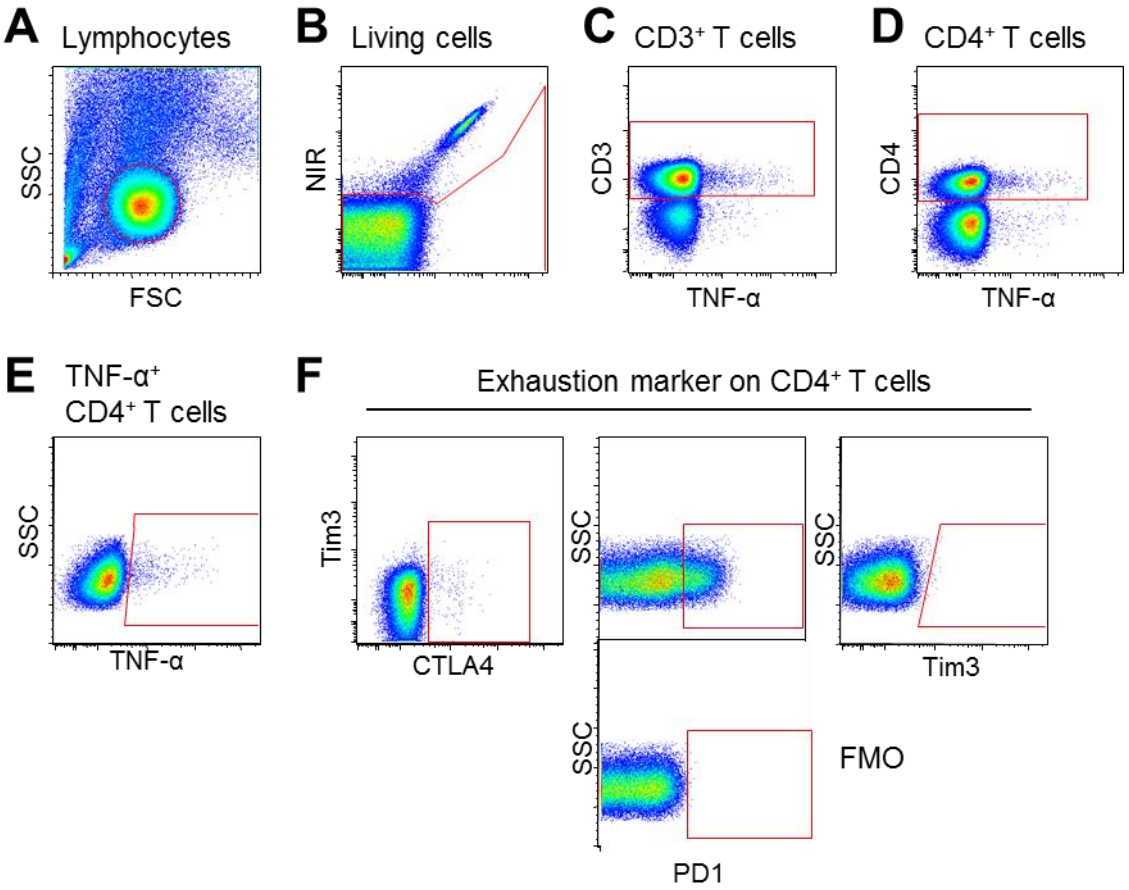



**Figure 4.17: Representative gating strategy for staining of exhaustion markers on $CD4^{TNF\text{-}mono}$ T cells.** Lymphocytes were gated based on FSC versus SSC plot (**A**), followed by exclusion of dead cells by NIR staining (**B**). CD3 (**C**) and afterwards CD4 cells (**D**) were gated against TNF-α. Once CD4 T-cell population was defined, CD4 T cells positive for TNF-α (**E**) and for exhaustion markers CTLA4, PD1 and Tim3 (**F**) were separately identified by using different plots in which the axis were chosen to provide the best discrimination between positive and negative events. PD1 was gated in the fluorescence minus one (FMO) sample.

### 4.3.7 $CD4^{TNF\text{-}mono}$ T cells do not express activation, exhaustion and $T_{reg}$ markers

As $CD4^{TNF\text{-}mono}$ T cells are predominantly antigen-experienced memory T cells, the activation status of these cells was analysed in patients with chronic hepatitis B. Despite TNF-α production of $CD4^{TNF\text{-}mono}$ T cells without *ex vivo* restimulation, 96.36% (Mdn.) of the cells did

not express T-cell activation markers 4-1BB, Ox40 and TNFR2 (Figure 4.18A). Next it was analysed if $CD4^{TNF\text{-}mono}$ T cells express the T-cell exhaustion markers PD1, CTLA4 and Tim3. Only a small proportion of $CD4^{TNF\text{-}mono}$ T cells expressed one or two of these exhaustion markers (Mdn: 13.36% and 0.66%, resp.), whereas most of the cells were negative for PD1, CTLA4 and Tim3 expression (Figure 4.18B).

Finally, it was determined if $CD4^{TNF\text{-}mono}$ T cells belong to the subpopulation of $T_{regs}$ as frequencies of these cells were described to be elevated in chronic HBV, HCV and HIV infection. The majority of $CD4^{TNF\text{-}mono}$ T cells expressed no or only one $T_{reg}$ marker (Mdn: 51.67% and 40.21%, resp.; Figure 4.18C). Expression of the most relevant $T_{reg}$ markers FoxP3 and CD25 in parallel was only observed in a minority of the cells. Taken together these results indicate that $CD4^{TNF\text{-}mono}$ T cells are antigen-experienced memory CD4 T cells that do not express activation or exhaustion markers and do not belong to the subpopulation of $T_{regs}$.

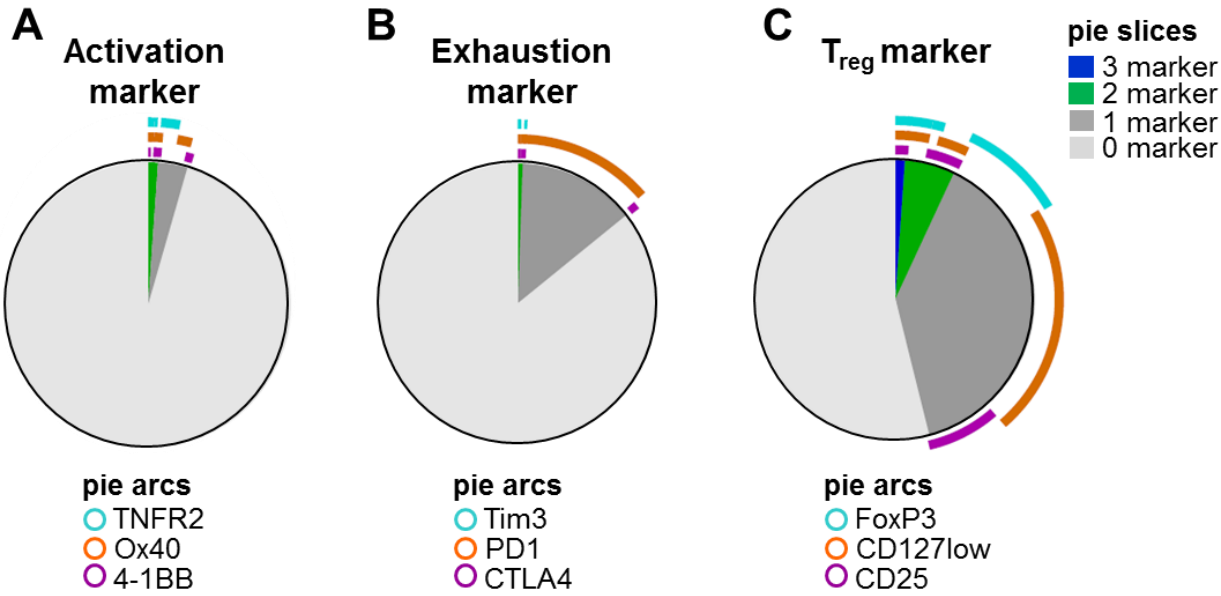



**Figure 4.18: $CD4^{TNF\text{-}mono}$ T cells do not express activation, exhaustion and $T_{reg}$ markers.** Frequencies of $CD4^{TNF\text{-}mono}$ T cells in patients with chronic hepatitis B without PEG-IFN-α treatment (n=8) were determined by flow cytometry without restimulation of PBMC *ex vivo*. $CD4^{TNF\text{-}mono}$ T cells expressing activation (A), exhaustion (B) and $T_{reg}$ (C) markers were depicted as frequencies (%) of total $CD4^{TNF\text{-}mono}$ T cells. Each pie slice corresponds to the proportion of $CD4^{TNF\text{-}mono}$ T cells positive for a certain combination of T-cell markers. Individual marker expression is depicted by the concentric colored arcs surrounding the pie chart. Color codes defining the number (slices) and type (arcs) of T-cell marker are indicated.

# 5 Discussion

## 5.1 Comparison of HBV-specific T-cell monitoring *ex vivo* and after a 10-days *in vitro* expansion

Monitoring of HBV-specific T cells was performed using HBc and HBs peptide pools including all described CD4 and CD8 T-cell epitopes. Optimized peptide pools that include only defined T-cell epitopes were described to induce stronger T-cell responses than overlapping peptide pools that typically consist of 15-mers that cover the whole protein [141]. One explanation might be that optimal T-cell epitopes do not have to be processed before they can be presented on MHC molecules. Additionally, optimized peptide pools do not include irrelevant peptides in non-antigenic regions that might lead to loss of T-cell responses due to competition among peptides. Further, it was already described that using increased numbers of peptides per pool inhibits the proliferation of CD8 T cells possibly by inducing T-cell exhaustion [142]. Usage of complete HBc and HBs protein instead of peptide pools would have the advantage of naturally processed peptides. However, exogenously ingested antigens do not enter the MHC I antigen presentation pathway and are therefore not able to induce CD8 T-cell responses.

Effective HBV-specific CD4 and CD8 T-cell responses are essential to control HBV infection and establish a protective humoral immune response [26]. Therefore clinical immune monitoring of HBV-specific T-cell responses in self-limiting and chronic hepatitis B is an important strategy to identify correlates of protection in order to develop new immunotherapies against persistent HBV infection. Several immunotherapeutic strategies to stimulate effective HBV-specific T cells in patients with chronic hepatitis B were developed and evaluated in clinical trials [143]. Monitoring of HBV-specific T cells is an important primary endpoint of clinical trials aiming to prove efficacy of T-cell inducing therapies in patients with chronic hepatitis B. Experimental procedures in clinical trials should be reported in detail according to the guideline *MIATA* and not changed during a study [144] [145]. As there is no internationally standardized clinical T-cell monitoring available data generated from different laboratories are difficult to compare and interpret. Hence there is a strong need for standardization and validation of methods to monitor HBV-specific T cells in international proficiency panels prior to their use in clinical trials. To analyse quantity and functional profile HBV-specific T cells sensitive, precise and robust methods are essential. A variety of assays including intracellular cytokine staining (ICS), enzyme-linked immunospot (ELISpot) and multimer staining is available for the monitoring of antiviral T-cell responses. The present work used the advantage of flow cytometry-based polychromatic ICS to analyse quantity and detailed functional phenotype of HBV-specific T cells in parallel [129]. Several experimental

parameters have been shown to impact marker expression of antigen-specific T cells hence analysis of T cells by polychromatic ICS requires standardized and validated experimental protocols [130-133]. As frequencies of peripheral HBV-specific T cells are very low in chronic hepatitis B patients *in vitro* expansion of PBMC for up to 14 days prior to a T-cell monitoring is commonly used to increase assay sensitivity. However, *in vitro* expansion of PBMC might change the HBV-specific T-cell population due to preferential expansion of particular T-cell clones. This would implicate that HBV-specific T-cell population may not fully reflect T-cell reactivity *in vivo*. Therefore one part of this work was the detailed analysis of quantity and quality of HBV-specific T-cell responses determined *ex vivo* compared to an analysis after a 10-day *in vitro* expansion phase.

As expected total HBc-specific CD4 and CD8 T cells as well as HBs-specific CD4 T cells were significantly increased in number after *in vitro* expansion. However, this hold not true for HBs-specific CD8 T cells which were detectable in only 16 out of 41 patients after *in vitro* expansion whereas 20 out of 41 patients had a detectable T-cell response *ex vivo*. The low proliferative capacity of HBs-specific CD8 T cells *in vitro* might be explained by their high activation threshold [146]. Another possibility might be an increased susceptibility to activation-induced cell death which was described for T cells with high T-cell receptor avidity [147]. Furthermore expression of the inhibitory receptor killer-cell lectin like receptor G1 (KLRG1) could explain the low proliferative capacity of HBs-specific CD8 T cells *in vitro* as this was associated with a lack of proliferative capacity [148]. Bengsch and coworkers observed expression of KLRG1 by HBV-specific T cells in patients with chronic hepatitis B. However, expression was lower compared to the inhibitory receptors PD1 and 2B4 [149]. Expansion of HBs-specific CD8 T cells might be improved by addition of anti-apoptotic cytokines like IL-7 and IL-15 to avoid T-cell receptor activation-induced cell death [151]. However, these approaches are useful to expand T cells for purposes like T-cell receptor analysis or T-cell therapy but not for clinical T-cell monitoring as they would be a further experimental parameter manipulating marker expression of HBV-specific T cells. Overall proliferative capacity of FEC-specific T cells *in vitro* was higher compared to HBV-specific T cells indicating a possible intrinsically hampered proliferative capacity of HBV-specific T cells. However, this can so far not be explained.

HBs-specific CD4 and CD8 T cells detectable *ex vivo* but not after *in vitro* expansion were mainly monofunctional. Compared to HBs-specific T cells that were detectable >2-fold increased after expansion these cells showed in particular decreased expression levels of IL-2. HCV-specific CD4 T cells of patients with chronic hepatitis C that produce IFN-γ but not IL-2 were associated with a low proliferative capacity *in vitro* [150]. Therefore one might assume that monofunctional HBs-specific T cells that lack IL-2 expression are more sensitive

to mechanisms that inhibit *in vitro* proliferation compared to polyfunctional T cells. Limiting amounts of IL-2 cannot be the reason for the low proliferative capacity of these cells as IL-2 was sufficiently added into the cell culture medium.

In contrast to acute HBV infection, chronic hepatitis B is characterized by a weak and monoclonal HBV-specific T-cell response due to T-cell exhaustion [26]. Hence one might assume that HBV-specific T cells from patients with chronic hepatitis B are less capable to proliferate *in vitro* compared to HBV-specific T cells from patients with acute hepatitis B as proliferation is the first function lost by exhausted T cells [80]. However, there was no significant difference in proliferative capacity of HBV-specific T cells *in vitro* between donors with acute and chronic hepatitis B. Furthermore there was only a weak correlation between frequencies of HBV-specific CD4 and CD8 T cells detectable *ex vivo* and after *in vitro* expansion in general. Taken together these data suggest that *in vitro* proliferation of HBV-specific T cells is neither related to the donor's HBV infection status nor to the *ex vivo* precursor frequency. Maini and coworkers already described that proliferative capacity of HBV-specific T cells *in vitro* is dependent on their activation status *in vivo*. Highly activated ($HLA\text{-}DR^{+}CD38^{+}$) HBc-specific CD8 T cells were associated with increased ALT levels and low proliferative capacity *in vitro* in patients with acute hepatitis B [64]. The low proliferative capacity of highly activated T cells is explained by an increased susceptibility to activation-induced cell death. The present results support these data as at least for HBc-specific CD4 T cells a strong negative correlation of proliferative capacity *in vitro* and serum HBsAg as well as ALT levels was observed in patients with acute hepatitis B. These observations indicate that highly activated HBV-specific T cells should be preferentially monitored *ex vivo* as they are potentially lost during *in vitro* expansion. It is quite conceivable that HBV-specific T cells that cannot be expanded *in vitro* reflect the *in vivo* situation. This suggests that patients with highly activated, poorly proliferating HBV-specific T cells would not respond to an immunotherapy that aims to induce proliferation of these cells. Therefore *in vitro* proliferative capacity of HBV-specific T cells might be a useful parameter in clinical trials to predict response to immunotherapies against chronic hepatitis B. To prove this hypothesis proliferative capacity *in vitro* should be determined and correlated with treatment response in immunotherapy trials.

Most studies monitoring HBV-specific T cells used *in vitro* expansion to increase assay sensitivity. However, in the present study the *in vitro* expansion only increased sensitivity of HBc-specific T-cell monitoring but not of HBs-specific T-cell monitoring. One possibility might be that HBs-specific T cells are less detectable after expansion due to loss of function. However, this hypothesis is not supported by the observation that sensitivity of HBs-specific T-cell monitoring after *in vitro* expansion was increased by measurement of multiple

cytokines (IFN-γ, TNF-α, IL-2) in parallel compared to single cytokine analysis. Further analyses are needed to identify the mechanism responsible for loss of HBs-specific T cells during *in vitro* expansion. Multimer staining of T cells allows their quantification independent of their functionality. Analysis of HBs-specific T cells *ex vivo* and after *in vitro* expansion by multimer staining could clarify whether HBs-specific T cells die due to activation-induced cell death or still lose their function due to exhaustion during *in vitro* expansion.

Beside the number of HBV-specific T cells also their functional profile changed after *in vitro* expansion. A correlation between the proliferative capacity *in vitro* and the degree of functional changes was observed. Therefore one can assume that the repertoire of HBV-specific T cells after *in vitro* expansion may not fully reflect the T-cell reactivity *in vivo*. One underlying reason for the modified functionality might be the preferential proliferation of certain T-cell clones *in vitro* due to antigen, costimulation, growth factor and nutrient competition or activation-induced cell death. For influenza virus-specific T cells it is known that dominant clones which preferentially consume IL-2 proliferate *in vitro* while subdominant clones stay in a resting state. This gradually leads to an oligoclonal T-cell population over time [152].

In summary, this work shows that *in vitro* expansion of PBMC strongly alters the number and functional (cytokine) profile of HBV-specific T cells. Precursor frequencies of HBV-specific T cells only weakly correlated with the numbers of cells determined after *in vitro* expansion and no general increase of assay sensitivity was observed after *in vitro* expansion of HBV-specific T cells. However, measurement of multiple cytokines (IFN-γ, TNF-α, IL-2) in parallel increased sensitivity of HBV-specific T-cell monitoring *ex vivo* and after *in vitro* expansion. The proliferative capacity of HBV-specific T cells *in vitro* might depend on T-cell activation status but not on *ex vivo* precursor frequency and the donor's HBV infection status. In conclusion *ex vivo* monitoring of HBV-specific T cells might reflect the *in vivo* T-cell reactivity best. As *in vitro* expansion is more time- and cost-consuming and more prone to inter-assay and -laboratory variability, a multiparametric (IFN-γ, TNF-α, IL-2) *ex vivo* analysis of HBV-specific T cells in clinical trials, especially when the functional profile of the T-cell response is examined, is suggested. Expansion may provide additional important information about proliferative capacity of HBV-specific T cells and treatment response in immunotherapy trials. Overall the present study revealed important knowledge for optimization, standardization and inter-laboratory comparability of clinical immune monitoring studies analysing HBV-specific T cells.

## 5.2 Phenotypic and functional characterization of $CD4^{TNF\text{-}mono}$ T cells

E*x vivo* monitoring of HBV-specific T cells in patients with chronic hepatitis B revealed a yet undescribed monofunctional CD4 T-cell subpopulation producing TNF-α but not IFN-γ, IL-2 or MIP1-β ($CD4^{TNF\text{-}mono}$) in the absence of antigen restimulation *ex vivo* [70]. Frequencies of $CD4^{TNF\text{-}mono}$ T cells were significantly elevated in patients with chronic hepatitis B and C, HIV mono- as well as HIV/HBV and HIV/HCV co-infection compared to healthy, HBV/HCV/HIV naïve individuals. Elevated $CD4^{TNF\text{-}mono}$ T-cell frequencies were also present in the acute phase of HBV infection but decline to levels of healthy HBV/HCV/HIV naïve individuals after resolution of HBV infection. Different studies described elevated levels of TNF-α in serum of patients with acute and chronic hepatitis B, chronic hepatitis C and HIV infection [153-156]. Increased production of TNF-α by PBMC has also been described in patients with fulminant and chronic hepatitis B and C [157, 158]. In line with the literature, the present data indicate that increased frequencies of $CD4^{TNF\text{-}mono}$ T cells might be associated with (chronic) virus infection. A monofunctional TNF-$\alpha^+$ (IFN-$\gamma^-$IL-$2^-$) *Mycobacterium tuberculosis (Tb)*-specific CD4 T-cell population was recently described to discriminate between latent and acute tuberculosis disease indicating relevance of these cells also in bacterial infections [159]. In contrast to tuberculosis infection $CD4^{TNF\text{-}mono}$ T cells associated with HBV, HCV and HIV infection produce TNF-α in the absence of antigen-specific restimulation *ex vivo* leading to the hypothesis that $CD4^{TNF\text{-}mono}$ T cells might represent a bystander T-cell population induced during viral infection.

To prove this hypothesis $CD4^{TNF\text{-}mono}$ T-cell lines were generated due to the low number of $CD4^{TNF\text{-}mono}$ T cells in peripheral blood. $CD4^{TNF\text{-}mono}$ T-cell lines generated from PBMC of patients with chronic hepatitis B lost their spontaneous TNF-α production stepwise during *in vitro* culture. Exhaustion of the cells due to continuous T-cell receptor stimulation might explain this phenomenon. PEG-IFN-α treatment of a $CD4^{TNF\text{-}mono}$ T-cell line for 4 weeks could not rescue spontaneous TNF-α production, although PEG-IFN-α treatment *in vivo* induced significantly increased frequencies of $CD4^{TNF\text{-}mono}$ T cells after 4 weeks in patients with chronic hepatitis B [70]. Furthermore, upregulation of TNF-α mRNA was observed in PBMC after PEG-IFN-α treatment *in vitro* [160]. $CD4^{TNF\text{-}mono}$ T-cell lines were used to analyse their antigen specificity. The T-cell lines did not react to restimulation with HBV antigens (HBs and HBc) *in vitro* indicating that $CD4^{TNF\text{-}mono}$ T cells are not HBV-specific. However, these results should be confirmed by $CD4^{TNF\text{-}mono}$ T cells stimulated with HBV antigens directly after sorting to exclude that T-cell unresponsiveness results from exhaustion of $CD4^{TNF\text{-}mono}$ T-cell lines during *in vitro* culture. High levels of soluble HBsAg typically circulate in patients with acute and chronic HBV infection. As it was shown that soluble HBsAg can efficiently induce T-cell

responses it is likely that HBsAg represents the priming source of $CD4^{TNF\text{-}mono}$ T cells *in vivo* [161]. However, HBsAg treatment was not able to induce TNF-α production by CD4 T cells of HBV/HCV/HIV naïve individuals *in vitro* supporting the hypothesis that $CD4^{TNF\text{-}mono}$ T cells are virus-unspecific bystander T cells.

To further gain insight which stimulus induced elevated $CD4^{TNF\text{-}mono}$ T-cell frequencies in patients with HBV, HCV and HIV infection, it was analysed if $CD4^{TNF\text{-}mono}$ T cells are cytokine-induced cytokine-producing T cells which were observed in different viral and bacterial infections and described as being unspecific T cells that produce cytokines induced by IL-18 [162-164]. *In vitro* IL-18 treatment was not sufficient to induce TNF-α production by CD4 T cells of HBV/HCV/HIV naïve individuals. PEG-IFN-α treatment induced elevated $CD4^{TNF\text{-}mono}$ T-cell frequencies in patients with chronic hepatitis B and C *in vivo* and upregulation of TNF-α mRNA in PBMC from healthy, HBV/HCV/HIV naïve donors *in vitro* [70] [160]. Hence, one might assume that type I IFNs induced by viral infections mediate elevated $CD4^{TNF\text{-}mono}$ T-cell frequencies in patients with HBV, HCV and HIV infection. However, PEG-IFN-α treatment *in vitro* did not support this hypothesis as no induction of TNF-α production by CD4 T cells of HBV/HCV/HIV naïve individuals was observed. It was already described that HBV might not induce type I IFNs in early HBV infection (incubation phase and log phase of viral spread) in chimpanzees [38]. Further no increased serum levels of IFN-α were observed in patients with acute hepatitis B at the time of peak viremia compared to a time point after resolution of HBV infection and healthy donors [37]. Hence in line with the literature the current data indicate that other factors than IFN-α are responsible for the initial induction of increased $CD4^{TNF\text{-}mono}$ T-cell frequencies.

PEG-IFN-α treatment of patients with chronic hepatitis B and C increased frequencies of already existing $CD4^{TNF\text{-}mono}$ T cells *in vivo* [70]. These data fit to the observation that PEG-IFN-α treatment induces upregulation of TNF-α mRNA in PBMC from healthy, HBV/HCV/HIV naïve donors *in vitro* as well as increased spontaneous TNF-α production by PBMC in patients with chronic hepatitis B *in vivo* [160, 165]. IFN-α could induce new $CD4^{TNF\text{-}mono}$ T cells by triggering TNF-α production in CD4 T cells. This might be mediated by members of NFAT and NF-κB nuclear factor families. It was shown that type I IFNs can directly activate these transcription factors which are then able to induce transcription of TNF-α gene [166] [167]. IFN-α may also induce proliferation of existing $CD4^{TNF\text{-}mono}$ T cells as type I IFNs promote T-cell expansion [168] [169]. Further studies analysing the gene expression profile of $CD4^{TNF\text{-}mono}$ T cells before and after PEG-IFN-α treatment *in vivo* are needed to define the detailed mechanisms how elevated $CD4^{TNF\text{-}mono}$ T-cell frequencies are induced.

Frequencies of TNF-α producing CD4 T cells were described to correlate with the degree of liver inflammation and fibrosis in patients with chronic hepatitis B [170]. Due to this important role of TNF-α producing CD4 T cells in chronic hepatitis B, this work aimed to phenotypically and functionally characterize $CD4^{TNF\text{-}mono}$ T cells associated with chronic HBV infection. The pro-inflammatory cytokine TNF-α exists in two isoforms - soluble and membrane-bound - with different affinities to its receptors TNFR1 and TNFR2. $CD4^{TNF\text{-}mono}$ T cells from patients with chronic hepatitis B secreted exclusively the soluble form of TNF-α which is preferentially bound by TNFR1. In contrast to TNFR2, TNFR1 can induce apoptosis in target cells [140]. $CD4^{TNF\text{-}mono}$ T cells might therefore play a role in TNF-α mediated killing of hepatocytes and thereby contribute to progressive liver damage in chronic hepatitis B. This is in line with data showing that T cell mediated TNF-α production and upregulation of TNFR1 expression on hepatocytes of patients with chronic hepatitis B play an important role in chronic liver injury [135, 136, 171]. The hypothesis that $CD4^{TNF\text{-}mono}$ T cells are involved in liver damage in chronic HBV infection is further confirmed by the observation that frequencies of $CD4^{TNF\text{-}mono}$ T cells correlated with serum ALT levels in patients with chronic hepatitis B receiving PEG-IFN-α add-on treatment [70].

Additionally, TNF-α can influence especially CD4 T-cell responses by inhibition of their proliferation and the negative regulation of Th1-like cells as wells $T_{regs}$ [172] [173] [137] [138]. Hence increased levels of TNF-α secreted by $CD4^{TNF\text{-}mono}$ T cells might also suppress HBV-specific T-cell responses as well as $T_{regs}$ which could further contribute to immunopathogenesis of chronic HBV infection. However, TNF-α also acts as suppressor of HBV replication. Therefore use of TNF-α inhibitors in patients with chronic hepatitis B can lead to decreased viral clearance presumably due to the failure of HBV-specific T cells to secrete appropriate amounts of TNF-α [174] [175]. In contrast to HBV-specific T cells $CD4^{TNF\text{-}mono}$ T cells seem to produce TNF-α independent of HBV antigen stimulation leading to unspecific killing of hepatocytes. Hence specific targeting of potentially liver damaging TNF-α produced by $CD4^{TNF\text{-}mono}$ T cells could be a promising strategy to treat chronic hepatitis B as beneficial TNF-α produced by HBV-specific T cells would remain unaffected.

TNF-α secretion is the first obtained effector function during maturation of an activated naïve T cell into a terminal effector T cell [176]. $CD4^{TNF\text{-}mono}$ T cells may represent naïve T cells that were activated by antigen recognition *in vivo*, but did not differentiate into terminal effector T cells. Phenotypical analysis of $CD4^{TNF\text{-}mono}$ T cells, however, revealed a mainly antigen-experienced memory phenotype which argues against an incompletely maturation status. As $CD4^{TNF\text{-}mono}$ T cells mainly represent antigen-experienced memory T cells, their activation status was analysed. T-cell activation is associated with the upregulation of TNF receptor family members. $CD4^{TNF\text{-}mono}$ T cells displayed no activated phenotype indicating that these

cells produce TNF-α independent from recent T-cell receptor activation [177]. The present data do also not support the hypothesis that $CD4^{TNF\text{-}mono}$ T cells represent exhausted T cells or belong to the subpopulation of regulatory T cells. Hence further analyses are required to characterize the function of $CD4^{TNF\text{-}mono}$ T cells. Importantly, the influence of $CD4^{TNF\text{-}mono}$ T cells on HBV-specific T-cell responses should be addressed in co-culture experiments. Additionally, their role in liver damage should be analysed in co-culture experiments with liver cell lines. Transcriptome analysis of $CD4^{TNF\text{-}mono}$ T cells is currently performed to characterize the gene expression profile of the cells which might provide conclusions about their phenotype and function.

In summary $CD4^{TNF\text{-}mono}$ T cells might represent a so far unknown bystander T-cell population in patients with HBV, HIV and HCV infection. Frequencies of $CD4^{TNF\text{-}mono}$ T cells increased by PEG-IFN-α treatment in patients with chronic hepatitis B and C but their initial trigger remains unclear. Due to the potential of TNF-α to suppress CD4 T cells and induce apoptosis in target cells, $CD4^{TNF\text{-}mono}$ T cells may contribute to immunopathogenesis of chronic viral infections. Hence, these cells might represent a new therapeutic target for treatment of chronic hepatitis B.

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# 7 Publications and meetings

## 7.1 Publications

**C. Russo**, C.J. Dembek, F. Geisler, M. Ringelhan, T. Michler, A. Umgelter, R. Draenert, U. Protzer, T. Bauer, *Impact of in vitro PBMC expansion on HBV-specific CD4 and CD8 T-cell monitoring in clinical trials*, submitted to Journal of Virology

**C. Russo**, M. Sprinzl, G. Fröschl, F. Geisler, A. Umgelter, J. Kittner, M. Schuchmann, R. Draenert, C. Geldmacher, U. Protzer, T. Bauer, *Role of monofunctional TNF-α producing CD4 T cells in chronic hepatitis B*, manuscript in preparation

J. P. Böttcher #, M. Beyer #, F. Meissner, Z. Abdullah, J. Sander, B. Höchst, S. Eickhoff, J. Rieckmann, C. Russo, T. Bauer, A. Dolf, E. Endl, D. Engel, S. Jung, D. Busch, U. Protzer, R. Thimme, M. Mann, C. Kurts, J. L. Schultze, W. Kastenmüller, P. A. Knolle, *$CX_3CR_1$/Fractalkine receptor expression separates memory $CD8^+$ T cells with distinct functional profiles*, submitted to Science

F. Bohne, M.C. Londoño, C. Benítez, R. Miquel, M. Martínez-Llordella, **C. Russo**, C. Ortiz, E. Bonaccorsi-Riani, C. Brander, T. Bauer, U. Protzer, E. Jaeckel, R. Taubert, X. Forns, M. Navasa, M. Berenguer, A. Rimola, J.J. Lozano, A. Sánchez-Fueyo, *HCV-induced immune responses influence the development of operational tolerance after liver transplantation in humans*, Science Translational Medicine, June 2014

M. F. Sprinzl, **C. Russo**, J. Kittner, S. Allgayer, A. Grambihler, B. Bartsch, A. Weinmann, P. R. Galle, M. Schuchmann, U. Protzer, T. Bauer, *Hepatitis B virus-specific T-cell responses during IFN administration in a small cohort of chronic hepatitis B patients under nucleos(t)ide analogue treatment*, Journal of Viral Hepatitis, November 2013

S. Kutscher, C.J. Dembek, S. Deckert, **C. Russo**, N. Körber, J. R. Bogner, F. Geisler, A. Umgelter, M. Neuenhahn, J. Albrecht, A. Cosma, U. Protzer, T. Bauer, *Overnight resting of PBMC changes functional signatures of antigen specific T-cell responses: impact for immune monitoring within clinical trials*, PLoS One, October 2013

C. J. Dembek, S. Kutscher, S. Allgayer, **C. Russo**, T. Bauer, D. Hoffmann, F. D. Goebel, J. R. Bogner, V. Erfle, U. Protzer, A. Cosma, *Longitudinal changes in HIV-1-specific T-cell quality associated with viral load dynamic*, Journal of Clinical Virology, July 2012

## 7.2 Meetings

2014 International Meeting on Molecular Biology of Hepatitis B Viruses

September 3-6, 2014; Los Angeles, USA

Oral presentation: Role of monofunctional TNF-α producing CD4 T cells in chronic hepatitis B

Poster presentation: Clinical immune monitoring: Impact of *in vitro* PBMC expansion on quantity and quality of HBV-specific CD4 and CD8 T cells

2014 Meeting of the German Association for the Study of the Liver

January 24-25, 2014; Tübingen, Germany

Poster presentation: HIV-1 infection impairs frequency and functionality of HBV-specific CD4 T cells in patients with chronic hepatitis B

2013 Meeting of the German Association for the Study of the Liver

January 25-26, 2013; Hannover, Germany

Poster presentation: Phenotypic characterization of circulating monofunctional TNF-$\alpha^+$ CD4 T cells in patients with acute and chronic hepatitis B

2012 International Meeting on Molecular Biology of Hepatitis B Viruses

September 22-25, 2012; Oxford, UK

Poster presentation: Monofunctional CD4 T cells producing TNF-α without antigen restimulation *ex vivo* dominate in patients with acute and chronic hepatitis B

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