**Fakultät für Medizin**
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# Altered HBV-specific T-cell immunity in HBV/HIV co-infection and chronic hepatitis B

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**Summary**

According to the WHO, 2.7 million people are persistently co-infected with both the hepatitis B virus (HBV) and the human immunodeficiency virus 1 (HIV-1). Clinical studies have demonstrated a higher risk for the development of chronicity and faster liver disease progression in HIV-1 co- compared to HBV mono-infection, but the immunopathogenesis remains unclear. Whereas HBV infects hepatocytes and leads to liver inflammation, HIV-1 infects immune cells like CD4 T cells. As HIV-1 needs their host cell to be activated, the infection itself induces immune-cell activation and eventually drives CD4 T cells into cell death. By contrast, T-cell immunity determines the natural history of the HBV infection. In fact, the acute infection is resolved in patients with a strong and polyclonal T-cell response, while the infection persists in patients with a weak and/or scarce T-cell response. The aim of this study was therefore to evaluate if HIV infection-mediated effects on T cells can alter HBV-specific T-cell immunity.

To elucidate the impact of HIV-1 co-infection on HBV-specific T-cell immunity, T-cell responses were compared with flow cytometry-based intracellular cytokine staining of peripheral blood mononuclear cells of 30 HBV/HIV-1 co- and 30 HBV mono-infected patients to determine cytokine (IFN-γ, IL-2, TNF) production of HBV core-, envelope- and polymerase-specific T cells. Frequencies of regulatory T cells (Treg), and T-cell exhaustion marker expression (e.g. PD-1, TIGIT) were determined. Exhausted T-cell populations were identified with datamining and clustering algorithms SPADE and t-SNE. The results were compared to data from HIV-1 mono-infected patients and healthy subjects.

While the frequency of HBV-specific T cells was comparable between HBV mono- and HIV-1 co-infection, CD8 T cells showed significantly different cytokine levels and cytokine expression profiles upon HBV protein-derived stimulation. This came in conjunction with differing levels of T-cell exhaustion markers between the cohorts. Clusters of terminally exhausted T cells were more prevalent in HBV/HIV-1 co-infection.

In summary, significant alterations of the cytokine expression of HBV-specific T cells of HBV/HIV-1 co-infected subjects are reported, possibly contributing to increased immunopathogenesis. The data indicate that HIV-1 co-infection is associated with increased CD8 T-cell exhaustion. This study therefore furthers the understanding of HIV-1 co-infection, which can give insight into the immunopathogenesis of HBV infection and HBV/HIV-1 co-infection alike, but also reveal key factors of spontaneous HBV clearance.

## Zusammenfassung

Laut der WHO haben 2.7 Millionen Menschen sowohl eine persistierende Hepatitis B Virus (HBV), als auch Humanes Immunodefizienz Virus 1 (HIV-1) Infektion. Klinische Studien zeigten höhere Risiken zur Entwicklung von Chronizität und schnellerer Fortschritt der Leberentzündung in HIV-1 Koinfektionen im Vergleich zu HBV Monoinfektionen, wobei die Immunopathogenese unklar bleibt. Während HBV Leberzellen infiziert, werden Immunzellen wie CD4 T Zellen durch HIV-1 infiziert. Das Ziel dieser Studie war daher herauszufinden, ob der Krankheitsverlauf von HBV Infektionen, welcher durch die T Zellimmunität des Wirts bestimmt wird, durch HIV-1 Infektion induzierte Immunzellaktivierung und -depletion in der HIV-1 Koinfektion beeinflusst wird.

Um den Einfluss der HIV-1 Koinfektion auf die HBV spezifische T Zellimmunität aufzuklären wurden periphere Blutmonozyten von 30 Patienten mit HBV/HIV-1 Koinfektion und 30 Individuen mit persistierender HBV Monoinfektion in Hinblick auf die Zytokinproduktion (IFN-γ, IL-2, TNF) von HBV core-, envelope- und polymerase-spezifischer T Zellen mittels Durchflusszytometrie basierter intrazellulärer Zytokinanalyse verglichen. Die Frequenzen regulatorischer T Zellen (Treg) wurden bestimmt und eine Evaluierung der T Zellerschöpfungsmarkerexpression wurde durchgeführt. Erschöpfte T Zellpopulationen wurden mit Gruppierungs- und Datenbankauswertungs-algorithmen SPADE und t-SNE identifiziert. Die Ergebnisse wurden mit Daten HIV-1 Monoinfizierter und gesunder Spender verglichen.

Während die Frequenz HBV spezifischer T Zellen zwischen Patienten mit HBV Mono- oder HBV/HIV-1 Koinfektion vergleichbar waren, produzierten CD8 T Zellen signifikant abweichende Zytokinmengen nach HBV protein-abgeleiteter Stimulation. Zusätzlich zeigten CD4 und CD8 T Zellen signifikant unterschiedliche Zytokinexpressionsprofile, sowie andere Ausprägungen von T Zellerschöpfungsmarkern. Gruppen terminal erschöpfter T Zellen waren in HBV/HIV-1 Koinfektion häufiger.

Zusammenfassend werden signifikante Abweichungen der Zytokinexpression HBV spezifischer T Zellen, die zur Immunpathogenese beitragen könnten, in HBV/HIV koinfizierten Patienten gefunden. Die Daten weisen auf eine Assoziation von HBV/HIV-1 Koinfektion mit erhöhter CD8 T Zellerschöpfung hin. Diese Studie erweitert daher das Wissen über HBV/HIV-1 Koinfektionen, welches Einblick in die Immunpathogenese der HBV Infektion gibt, jedoch ebenso Schlüsselfaktoren spontaner HBV Ausheilung identifizieren kann.

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## 1. Introduction

HBV/HIV co-infection burdens about 2.7 million people especially in Sub-Saharan Africa, but also the other regions of the world [1]. Clinical difficulties for co-infected patients mainly arise due to the interplay of the pathogenesis related to the hepatitis B virus (HBV) and human immunodeficiency virus (HIV) infection [2].

Both infections rely on the immune system. While the natural history of hepatitis B is greatly influenced by the host's immune response, HIV mainly infects immune cells [3, 4]. The introduction therefore starts with a short overview of the immune system and its mechanisms and cells.

The second part of the introduction explains the background of hepatitis B and the causative agent HBV. HBV infection is mostly restricted to hepatocytes, which are the main population of liver cells. The liver is one of the most important organs regulating metabolism, immunology and filtering the blood and degrade harmful substances. Many infectious agents are therefore trafficked through the liver or cleared by the organ what makes it more susceptible to infections than other organs. Additionally, blood clotting agents are synthesized in the liver and bile is produced that is secreted to the intestine. [5] The course of HBV infections is determined by the immune system [3]. While a functional immune response leads to the resolution of the infection, insufficient T- and B-cell immunity destines the acute HBV infection to progress to persistence [6].

In the third section of the introduction, the intricacies of HIV/AIDS are explained. HIV infects cells of the immune system like macrophages and CD4 T cells [4, 7, 8]. Macrophages residing in the tissue phagocytose pathogens and attract e.g. dendritic cells, which process and present foreign antigens to T cells in the lymph nodes [9]. This interplay leads to activation of T-cell immunity, which can be impaired by HIV infection [10].

In the fourth chapter, light is shed on the immunological and immunopathogenic knowledge in HBV/HIV co-infection as HIV infection can greatly impact the T-cell immunity-dependent course of HBV infections [2].

Finally, the aim of this study is defined and explained.

## 1.1 Molecular and cellular aspects of the immune system

### 1.1.1 Innate Immunity

Before induction of the adaptive immune response, the body relies on the functions of the innate immune system to fight pathogens like viruses. The innate immune system can recognize viral RNA, mostly the double-stranded RNA (dsRNA) that is found as a replication intermediate in most viral life cycles. The viral RNA is recognized by pathogen recognition receptors (PRRs) like TLR3, RIG-I and Mda5, which are essential for the activation of MAVS that triggers the expression of host-cell genes like *NF-κB*. NF-κB in turn induces the production of anti-pathogenic proteins like type I interferons upon identification of PAMPs (pathogen-associated molecular patterns) and DAMPs (damage-associated molecular patterns). PRRs are found on the surface of DC (dendritic cells), macrophages, monocytes, neutrophils and epithelial cells. [9, 11] The viral replication can either be antagonized by degrading the double strand and binding a complementary strand to the single-stranded RNA (ssRNA) or by inducing the type I interferons IFN-α and IFN-β that activate the JAK/STAT pathway, which in turn activates ribonucleases degrading the ssRNA. Host cells can also shut down the protein synthesis upon activation of the JAK/STAT pathway and thereby stop the translation of viral proteins until the viral genome is degraded. [9, 12-15]

Macrophages residing in immunogenic tissue are the cells that come into contact with invaders first. Recognition of TLRs, components of the complement system or antibodies on the surface of pathogens lead to actin polymerization and thereby allow macrophages to engulf the pathogen and start phagocytosis. After the pathogen is phagocytosed, macrophages continue to guard the tissue. The inflammatory response in form of the chemokine release by macrophages attracts more immune cells like monocytes and dendritic cells. [9, 16, 17]

Dendritic cells take up antigen and home to lymph nodes, where they prime lymphocytes and thereby initiate the adaptive immune response. The inflammatory response also causes fever and blood clotting to impede the spread of the pathogen. If the immune response gets out of control, it can trigger strong adverse effects like sepsis, autoimmunity or immunopathogenesis, which can result in the destruction of tissue. [9, 18]

The regulatory signal molecules, cytokines and chemokines, can lead to the activation, recruitment and inhibition of immune cells and are also able to directly target infected cells and affect them. Most interleukins are produced by monocytes, macrophages, endothelial cells, and helper CD4 T cells, which belong to the adaptive immune system. Interleukins can promote the differentiation of cells and are therefore important for the innate and the adaptive immune system. IL-2 induces self and non-self proliferation and stimulates the differentiation of T cells and antibody production of B cells, while IL-4 is released by CD4 helper T cells inducing the B-cell class switch. IL-6, mainly produced by macrophages and epithelial cells, induces B-cell differentiation and acute phase reactants in liver cells. Produced by monocytes, T-helper cells and regulatory T cells upon PD-1 signaling, IL-10 inhibits the production of cytokines like IFN-γ, IL-2 and the tumor necrosis factor (TNF). TNF can be produced by nearly every immune cell and induces fever, inflammation, and inhibits viral replication. [9, 19]

Interferons like IFN-γ are mainly produced by T cells and NK cells and further activation by sensitization of target cells to increase antigen processing and presentation. The cytokine also affects immune cells directly or indirectly by increasing MHCII expression. IFN-α is an antiviral cytokine mainly produced by DCs and induces the expression of ISGs like OAS1, Mx1 and ISG15 or ISG20. [9, 19, 20]

### 1.1.2 Adaptive Immunity

In contrast to innate immunity, adaptive immunity is very specialized and can give long-lasting protection. The adaptive immune system is activated by dendritic cells, which belong to the branch of innate immunity, that phagocytose pathogens and home to lymph nodes, where they induce B cells to release antibodies or present pathogen-derived antigens with MHC molecules to T lymphocytes. Upon activation, T lymphocytes migrate to the infection side and mount an inflammatory response. [9, 18]

B cells are lymphocytes releasing a wide variety of antibodies upon activation. They develop in the bone marrow (in adults) or the liver (in fetuses) from pluripotent hemopoietic stem cells. B cells can be activated upon protein binding or cross-primed by CD4 T cells. The produced antibodies can bind to antigens and thereby neutralize pathogens like viruses.

Antibody-bound antigens can also be targeted by macrophages and thereby be phagocytosed. Antibody-dependent cellular cytotoxicity (ADCC) is characterized by antibodies that bind antigens on the surface of e.g. infected or tumor cells, which can in turn be bound by Fcγ-receptors on immune cells like natural killer cells, which are thereby triggered to release cytotoxic molecules. Antibodies can also serve as a bridge for T cells to be activated when they bind the Fc domain (referred to as T-cell engagement). Additionally, B cells can present antigen to T cells. [9, 21-24]

T lymphocytes, also called T cells, develop in the thymus from precursors, which originated in pluripotent hemopoietic stem cells. T cells are mainly identified by bearing the CD3 surface protein, which is part of the so-called T-cell receptor (TCR). The TCR consists of the TCRα, TCRβ, CD3γ, CD3δ, and two CD3ε, as well as the intracellular CD3ζ chains. The TCRα- and β-chains define the antigen-specificity of the T cell, which arises due to somatic V(D)J recombination. By contrast, a minority of T cells expresses TCRγ- and δ-chains. There is a wide variety of differing T-cell types, which are functionally distinguished by being effector or memory T cells. Memory T cells are antigen-experienced but inactive T cells that are saved during the contraction phase of the immune response and can re-expand upon antigen stimulation. T cells are distinguished mostly dependent on their localization into central memory ($T_{CM}$, CD45RA$^-$ CCR7$^+$), effector memory ($T_{EM}$, CD45RA$^-$ CCR7$^-$), terminally differentiated effector memory ($T_{EMRA}$, CD45$^+$ CCR7$^-$), tissue-resident memory ($T_{RM}$, CD45RA$^+$ CCR7$^-$ CD69$^+$) and memory stem ($T_{SCM}$, CD45RA$^+$ CCR7$^+$, CXCR3$^-$ ) T cells. [9, 25-28]

Additionally, T cells can be differentiated by their expression of the CD4 and CD8 markers. CD4 T cells are generally referred to as T helper cells, whereas there are other CD4 T-cell subtypes, and CD8 T cells are called cytotoxic T cells. [9, 29-31]

T helper cells are mainly identified by their CD4 expression and CD154 (CD40L) co-expression and separated into different subtypes. $T_h1$ cells like other T cells are activated by IL-2 and regulated by the transcription factor T-bet to release IFN-γ and IL-2. $T_h2$ cells secrete immune-regulatory cytokines like IL-4 or IL-10, while Tfh (follicular helper T) cells, which reside in the lymph nodes, are known to prime B cells. [9, 32-35]

Cytotoxic T lymphocytes are predominantly CD8 T cells and kill infected cells by the release of perforin, which forms pores in the infected cells, rendering them vulnerable to

simultaneously secreted proteases like granzyme B, which activate Caspase-mediated apoptosis. Alternatively, Fas ligand is released and induces caspases upon binding its fas receptor. CD8 T cells are activated upon T-cell receptor-mediated recognition of peptide-loaded MHCI complex on antigen presenting cells (APC), which results in the secretion of cytokines like IFN-γ and TNF. The TCR – MHCI interaction is dependent on the co-stimulation with CD8 and leads to the activation of ZAP70 and the downstream release of IP3 and DAG by PLCγ, which results in the $Ca^{2+}$ – calcineurin-dependent activation of the transcription factor NFAT. The interaction of NFAT and AP1 (which consists of Fos and Jun subunits and is a transcription factor triggered in a RAS-dependent manner upon TCR activation) is required for a functional T-cell response. T cells can proliferate upon antigen recognition and co-stimulation with IL-2. The activation mostly occurs at the site of infection or in the lymph nodes. [9, 29-31, 36-42]

Chronic infectious diseases are often characterized by T-cell dysfunction [43-45]. This defines a state in which T cells do not function properly and cannot control the infection. Mechanisms of T-cell dysfunction are quite heterogeneous and include antagonization of antiviral T-cell responses by anti-inflammatory cytokines or regulatory T cells, as well as the expression of inhibitory receptors, inadequate antigen-processing or presentation and a transcriptional reprogramming [45]. Failing T-cell responses are often linked to unbalanced NFAT – AP1 interaction in the T-cell nucleus and can lead to apoptosis. [9, 46]

Regulatory T cells are T lymphocytes that mainly suppress the function of other T cells. They bear the CD4 receptor and express the CD25 receptor and the FoxP3 transcription factor, but lack the expression of CD127. Treg differentiation from naïve T cells is induced upon stimulation with TGF-β. They are able to express the cytokine IL-10 to inhibit cytotoxic CD8 T cells [9]. Treg are important to reduce immunopathogenesis and the incidence of autoimmunity [47].

T-cell exhaustion is a status of T cells that is characterized by a progressive loss of cytokine secretion and identified by the upregulation of inhibitory markers [45]. The cause of T-cell exhaustion is controversial with hypotheses pointing at either a superfluous amount of antigen causing hyperactivation, an immunosuppressive microenvironment or a constant level of antigen leading to prolonged activation [48-53]. Recently, T-cell exhaustion was

identified as an independent T-cell phenotype, which is distinguished from the memory phenotype by epigenetic programming [54].

In persistent viral infections, T-cell exhaustion plays a major role. It is described for CMV, EBV, HIV, HCV and also HBV infection [43]. Since persistent infections cannot be cleared by the immune system, possible options for the rescue of exhausted T cells e.g. by early IL-2 treatment are tested [55, 56]. Expression levels of CD127 and PD-1 were used to define different T-cell subsets and populations of exhausted T cells. Terminally exhausted T cells have a phenotype of CD127$^{-}$ PD-1$^{high}$ Tcf-1$^{-}$, cannot proliferate and are irreversibly functionally impaired. CD127$^{+}$ PD-1$^{+}$ Tcf-1$^{+}$ is the phenotype of so-called memory-like, stem-cell like, precursor or progenitor exhausted T cells [57-61]. These cells can still proliferate and are able to replenish the T-cell population e.g. after treatment success [62]. The presence of these Tcf-1$^{+}$ precursor T cells is required for effective PD-1 blockade and therapeutic vaccination [63, 64]. But T-cell exhaustion does also inhibit the success of immunomodulatory treatment like adoptive T-cell transfer [65], although it was recently shown that adoptively transferred T cells can be made resistant to T-cell exhaustion by *c-Jun* overexpression [66].

In this study, multiple markers are analyzed to assess the status of T cells and to identify T-cell exhaustion. These markers are presented below.

CD127 is the IL-7 receptor α-chain and was found to be important in lymphocyte development. The cytokine IL-7 is important for the differentiation of hematopoietic stem cells to lymphocyte progenitors and for the proliferation of lymphocytes [67]. CD127 is therefore central to the replenishment of antigen-specific T cells and can be found on e.g. memory T cells. It is not present on Treg and its expression inversely correlates with FoxP3 expression [68]. In stable or well recovering HIV patients, CD127 expression was found to be correlated with CD4 counts [69].

PD-1 (programmed cell death protein 1), also called CD279, is physiologically important for the inhibition of autoimmunological T cells and thus regulates inflammation [70]. It is therefore naturally expressed in activated T cells, while very high levels indicate dysfunctional T cells. PD-L1 (PD-ligand 1) can either bind the costimulatory molecule CD28 or PD-1 and has therefore both inhibitory and activating functions [71]. Nevertheless, PD-1 also triggers the production of the inhibitory cytokine IL-10 by CD4 helper and regulatory cells or monocytes [72, 73]. Overall, it was found that interaction of PD-1 with PD-L1 inhibits

proliferation, activation and the IFN-γ and IL-2 secretion of virus-specific CD8 T cells [74, 75]. T cells with high levels of PD-1 are therefore associated with T-cell exhaustion. The exhaustion marker CTLA-4 is a paralogue to the activation marker CD28 as both bind to CD80, and CTLA-4 can thereby antagonize T-cell activation. Similarly, TIGIT is a paralogue to CD226 with the binding partners CD155 and CD112 [76, 77]. TIGIT inhibits T-cell activation by release of IL-10 and inhibition of IL-12 [78], is found on Treg [79] and exhausted CD8 T cells [80]. CD39 was found on exhausted T cells in HIV [81] and on CD4 Treg in HBV infection [82]. While its expression seems to suppress T-cell survival [83], it is important for T-cell homeostasis and the differentiation of helper T cells [84].

The transcription factors T-bet, Eomes and Tcf-1 are used to distinguish exhausted T cells from memory or cytotoxic T cells. T-bet is a transcription factor crucial for the development of T cells to IFN-γ secreting subtypes. Eomesodermin is the paralogue of T-bet and was found to stop CD8 T-cell differentiation [85]. Tcf-1 is a transcription factor for the *CD3e* gene and was found to be one of the central transcription factors (with Gata3 and Bcl11b) in the maturation of all T cells. In studies characterizing T-cell exhaustion, Tcf-1 was found to be present in the precursor memory-like T cells, but to be absent in terminally exhausted T cells [86]. Since 2019, the transcription factor Tox was also found to play a major role in the exhaustion of CD8 T cells [87-89].

## 1.2 Hepatitis B

### 1.2.1 History of hepatitis B and major research breakthroughs

Hepatitis B is a liver inflammation that is caused by the infection of liver cells with the hepatitis B virus [90]. Lately, HBV genomes were discovered in ancient human samples that are thought to be 800 to 4 500 years old. The genesis of the first hepatitis B virus is estimated to be 8 600 to 20 900 years ago [91, 92]. Hepatitis B was first described as serum hepatitis in 1885 by clinician Lurman after the disease was spread in an attempt to vaccinate against smallpox in Bremen, Germany in 1883 [93]. Frederick MacCallum hypothesized in 1947 the cause of the disease to be a virus [94]. In 1966, Baruch Blumberg (being awarded the Nobel prize in Physiology and Medicine in 1976) discovered the HBsAg (then known as the Australia Antigen) in human serum [95], but this was only connected to serum hepatitis in 1968 by Alfred Prince [90] and only a few years later, in 1970, David Dane discovered the virus particle by electron microscopy [96]. In 1979, the HBV genome was sequenced [97].

The first approved HBV vaccine was developed by Maurice Hilleman and came to market in the US in 1981 as Heptavax [98], while the recombinant vaccine containing HBsAg grown in yeast was deployed from 1986, developed by Pablo DT Valenzuela [99]. In 1992, the WHO called on all states to enforce an HBV vaccination in their routine vaccination schemes. The vaccine recommendation for all children was incorporated to RKI (by the Ständige Impfkommission (STIKO)) and the CDC in 1995 and 1997 respectively [100].

### 1.2.2 Hepatitis B Virus

#### 1.2.2.1 Phylogeny, genome and structure

The hepatitis B virus belongs to the family of Hepadnaviridae and the genus of Orthohepadnaviruses. Other viruses in the genus are Woodchuck hepatitis virus, Woolly monkey hepatitis B virus, Ground squirrel hepatitis virus and four bat hepatitis B viruses (Long-fingered bat, Pomona bat, Roundleaf bat, Tent-making bat). In the family of Hepadnaviridae, there is only one other genus which is Avihepadnaviruses consisting of the Duck hepatitis B virus, the Heron hepatitis B virus and the Parrot hepatitis B virus. [101]

Viral relatives were found to be nackednaviruses, which seem to have common ancestors with HBV [102].

The hepatitis B virus is part of group VII in the Baltimore scheme containing viruses replicating their dsDNA (double-stranded DNA) genome dependent on reverse transcriptase. Only Baltimore group VI is also dependent on reverse transcriptase, but bears viruses with ssRNA (single-stranded RNA) genome. This group consists of retroviruses and the lentiviruses with its prominent member HIV-1. [101, 103]

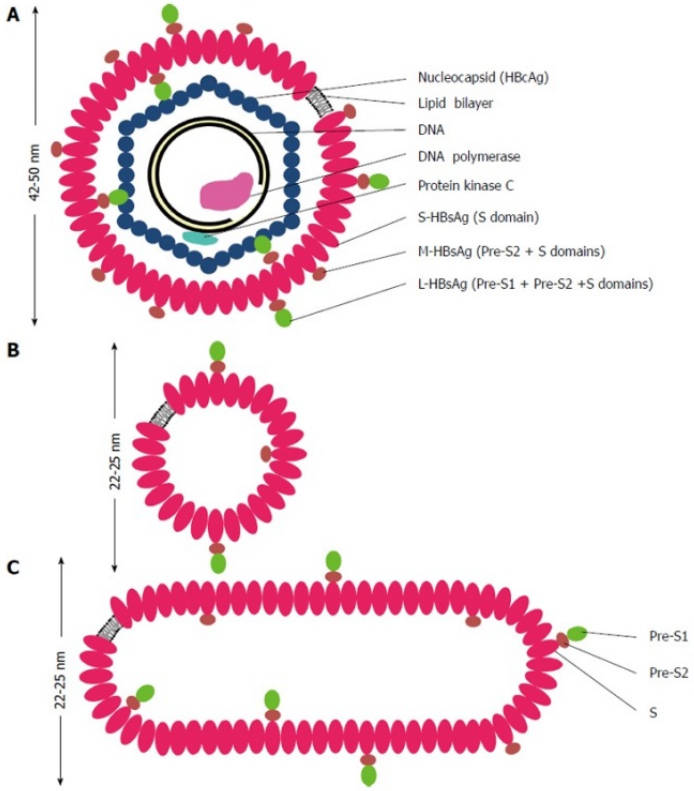


Figure 1: **Structure of HBV virions and subviral particles** [104]. A) An infectious hepatitis B virion, the so-called Dane particle is shown consisting of the lipid bilayer associated with HBsAg enveloping the icosahedral capsid, which contains the rcDNA, protein kinase C and the DNA polymerase. Additionally, HBV infected cells produce B) spherical and C) filamentous subviral particles.

The hepatitis B virus is an enveloped virus with a double-stranded DNA genome (Figure 1). The DNA is bound to the polymerase. Both are found in an icosahedral capsid that is enveloped by a lipid membrane. In general, infectious HBV particles are called Dane particles, but there are also subviral particles which are filamentous or spherical. Besides recognizing infectious virions, the immune system can also be stimulated by uninfectious viral particles. Dane particles have a diameter size of 42 nm, while the spherical subviral particles have 22 – 25 nm and the filaments vary in size [105, 106]. The envelope consists of phospholipids, lipoproteins and viral proteins, while the HBV env protein is found on the membrane. The normal HBV capsid consists of 240 copies of HBc protein in an icosahedral T = 4 shape. Another variant of the capsid that is quite rare is composed of 180 copies of HBc in an icosahedral T = 3 shape. Inside the capsid, the genome is found as the relaxed circular DNA (rcDNA), which has a complete minus strand, but incomplete plus strand. [101]

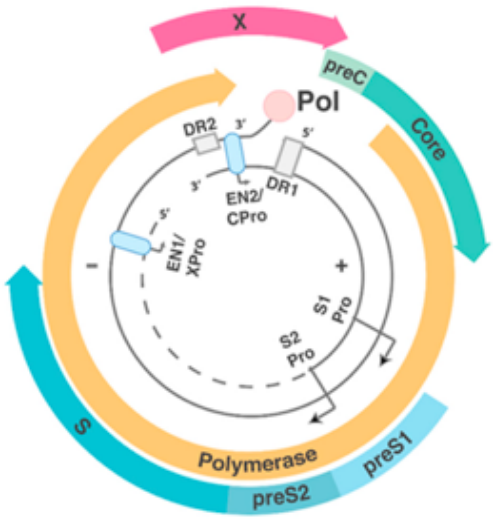


Figure 2: **HBV genome and ORFs** [107]. The HBV genome is a doublestranded nicked rcDNA and consists of four overlapping open reading frames encoding for the preS1, preS2, S, X, preCore, Core and polymerase transcripts. There are four promotors as indicated on the scheme of the rcDNA. The 3' end of the negative strand is associated with one polymerase molecule.

The HBV genome is 3020 – 3320 nt/1700 – 2800 nt long and is completed by cellular DNA polymerases. The resulting episomal covalently closed circular DNA (cccDNA) is instable upon cell division, but non-proliferating hepatocytes or hepatocytes with an integrated HBV genome save the genome from being lost. It encodes four overlapping open reading frames (ORF) increasing the coding capacity of the genome immensely (Figure 2). Four promotors

(core, pre-S1, pre-S2/S and X) are starting the transcription of the nine transcripts with the help of two enhancer regions ENH1 (activating the early transcription of the X mRNA) and ENH2 (late transcripts pgRNA, pcRNA and S mRNA) [108]. Seven proteins are produced from the HBV genome. [101, 109]

Spanning the gap of the incomplete dsDNA is the ORF coding for the X protein, which seems to be the regulator of transcription and which is only transcribed and translated when the incomplete rcDNA is successfully converted to the cccDNA. The x promotor is regulated by the nuclear respiratory factor 1 (NRF1), which activates mitochondrial genes [110]. The HBx mRNA is transcribed early and accumulates about 16 hours after transfection and is not found after four days [111, 112]. There are three types of transcripts of the X promotor resulting in the X protein's mRNA of 0.7 kb, small transcripts [113] and a controversial overlength form of 3.9 kB being transcribed if the polyA signal is skipped, which may happen due to an activated state the cellular polymerase is in [114, 115]. This overlength HBx has no known function and is not regularly exported from the nucleus [108, 112]. Nevertheless, another long HBx transcript was found in 2020 [116]. All other transcripts are viewed to be late transcripts and are detectable about 20 hours after transfection. [101, 109]

The core promotor region starts the transcription of the precore mRNA (3.4 kb), which is translated to the precore protein (p25) that is proteolytically cleaved at the N and C termini to generate the HBeAg (p17). Due to leaky scanning, the transcription of the pregenomic RNA (pgRNA, 3.5 kb) can start in the core promotor region just downstream of the precore mRNA [117, 118]. A stop codon 300 bp downstream from the start codon in the core promotor region is ignored by the cellular polymerase when reading it for the first time, but is recognized when the transcript has reached 3.5 kb. This is accounted to the activated state of the cellular polymerase in the beginning [115]. From the pgRNA the core protein is translated and it is the template for the generation of the rcDNA by the reverse transcriptase. Alternative splicing of the pgRNA leads to the hepatitis B spliced protein (HBSP) that has no known function. [119] The preCore and core mRNA encode for the core and excretory protein which are recognized as the HBcAg and the HBeAg. The core proteins form the virions nucleocapsid, while the excretory protein has an immune evasion function as it downregulates TLR-2 on hepatocytes. As HBeAg is found in the serum, it can be used as a peripheral marker for the hepatic HBV replication. It can further be used to underline the infection's diagnosis and evaluate therapy success. [101, 120]

From the pgRNA ORF, the Polymerase mRNA (which is the biggest mRNA) is produced by leaky scanning [121-124] encoding a single protein that has a terminal protein domain at the N-terminus and spacer domains, the reverse transcriptase and a C-terminal RNaseH function and is a type III polymerase, which do not recognize specific termination structures [101, 109].

Overlapping strongly with the Polymerase ORF is the preS1, preS2 and Surface ORF that encode the small, medium and large surface proteins of the HBV particles. Two promotor regions, the preS1 and the S promotor are found on the genome. The preS1 promotor is found upstream of the preS1 mRNA (2.4 kb; its transcription is regulated by the hepatocyte-specific transcription factor HNF1alpha and its cofactor Oct-1) encoding for the large surface protein (L). The second promotor, the S promotor produces the transcripts for the medium (M) and small (S) HBsAg. [101, 109] The promotor is regulated by ubiquitous transcription factors, RXR-alpha and HNF-4 [108, 125, 126]. For one S transcript, 5 preS mRNA are produced [127]. A 2.1 kB transcript is liver- and kidney-specific and maybe dependent on HNF-3 [128]. The serum concentration of HBsAg is viewed as a correlate of the active cccDNA in the liver [129]. Too low HBsAg can be observed very early in the infection, during acute or occult infection. Escape mutations of HBsAg can lead to false negative results in diagnostics due to low antibody binding capacity to the mismatch. To confirm the infection, highly sensitive detection of HBV DNA is performed. [130]

HBV mutation rate is estimated to be $8.04\times10^{-6}$ – $1.51\times10^{-5}$ nucleotide substitutions per site every year [91]. Particles with a strong drift in their genomes can be assigned to different quasispecies, which are due to the overlapping ORF mostly found in the X and the Core ORF [131]. The hepatic tropism is thought to be strict due to transcriptional restrictions [132].

#### 1.2.2.2 HBV Life Cycle

The hepatitis B virus attachment and entry of hepatocytes is mediated by heparan sulfate proteoglycans and the bile acid receptor (NTCP) [133] (Figure 3). After the entry during the uncoating, HBV particles shed their envelope and are transported to the nucleus. At the nucleus membrane, the genome is released to the nucleus and the rcDNA is converted to

cccDNA. This conversion may be dependent on DNA repair mechanisms of the host cell possibly in PML nuclear bodies since SUMOylated HBc colocalizes with PML nuclear bodies. [101, 134, 135]

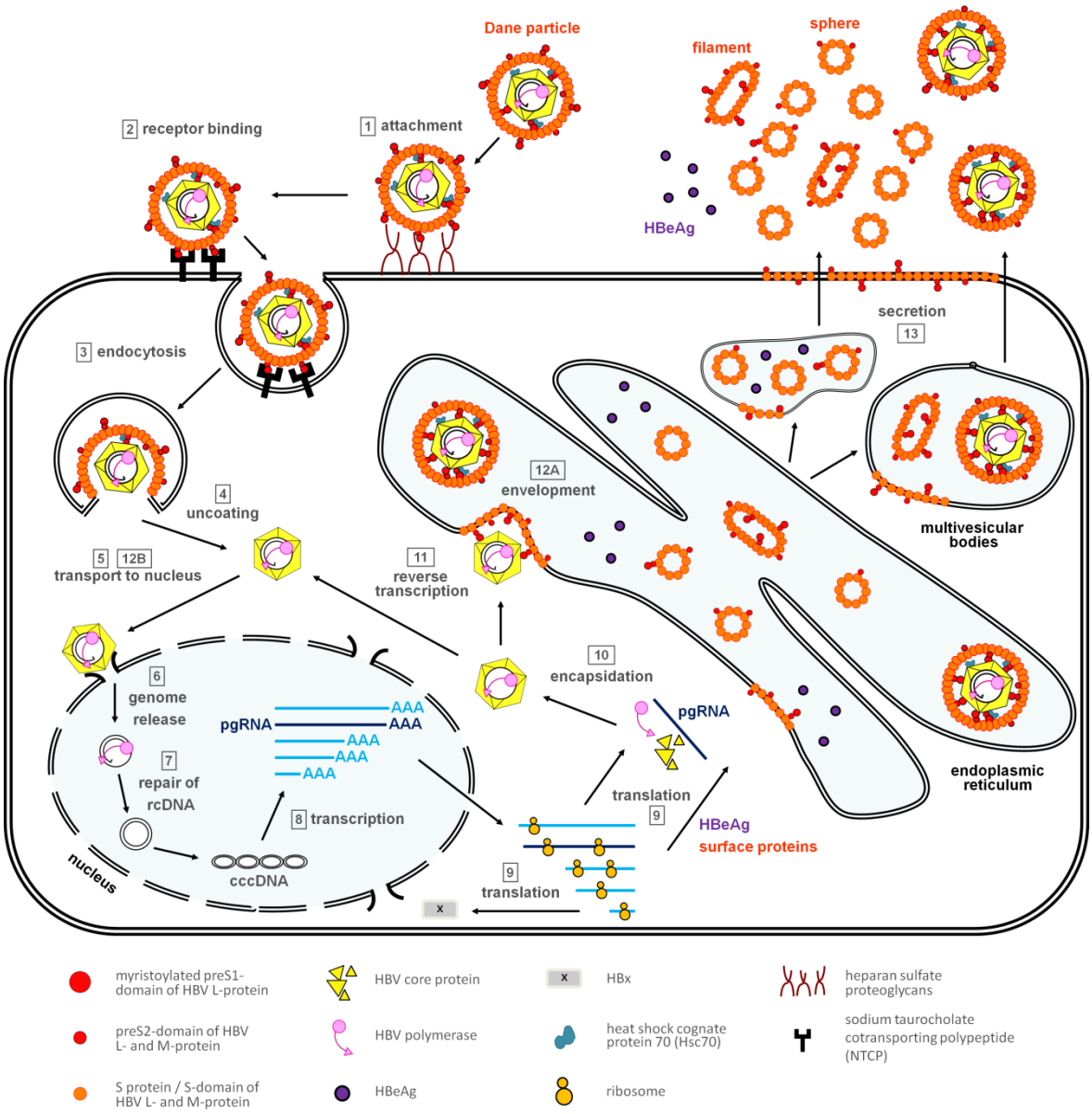


Figure 3: **HBV life cycle** [136, 137]. 1) Dane particles attach to proteoglycans on the cell membrane of hepatocytes. 2) Virion interacts with NTCP and 3) becomes endocytosed. 4) The capsid becomes uncoated by fusion of the lipid envelope with the endosome and 5) is transported to the nucleus, where 6) the rcDNA genome is released and 7) repaired to cccDNA. The endosomal cccDNA is 8) transcribed to pgRNA and the subgenomic RNAs, which are shuttled from the nucleus and 9) translated to the viral proteins. The HBeAg and the HBsAg are shuttled via the ER to the cell membrane. The polymerase associates to the pgRNA and is 10) encapsidated by core proteins, while 11) the RT function of the polymerase reverse transcribes the pgRNA to rcDNA in the capsid. The capsids are either 12A) enveloped by entering the ER or 12B) re-enter the nucleus. Enveloped viral particles are shuttled through the ER and 13) secreted from the hepatocyte.

cccDNA exists in the nucleus in parallel to the hosts genome as a so-called episome which can be used as a replication blueprint. The replication can be regulated by histone modification of the cccDNA chromatin. Besides, HBV DNA is also able to integrate randomly into the host genome. From these two sources of HBV genome states mRNA is transcribed by the host cell machinery via RNA polymerase II. Spatial organization in the nucleus may be a factor for the transcription machinery. [101, 109] Controversially, HBx may be a regulator of transcription from cccDNA [138, 139]. CpG islands regulate the transcription besides the factor Cfp1 that is recruited to the cccDNA and required for transcription [140]. The hepatocyte transcription factors HNF-4α and HNF-1α, which are more strongly expressed upon further hepatocyte differentiation, are needed for HBV replication and the transcription of the HBV pgRNA [132] and the cccDNA [141].

Most of the HBV transcripts are translated to the viral proteins by the host cells ribosomes, except for the pgRNA that is the genome precursor. While the X protein returns to the nucleus, the core, envelope and polymerase proteins are used for the assembly of the virions. After encapsidation of the pgRNA template with the core protein, the RNA is reverse transcribed by the RT-function of the HBV polymerase to rcDNA, the capsids can either reenter the nucleus and thereby provide more transcription and replication templates or are enveloped at the endoplasmatic reticulum and transported via the multivesicular bodies and golgi route to the cell membrane. In parallel, the HBeAg and the envelope proteins can also be independently transported to the membrane, while subviral particles consisting of a host cell's lipid layer with the viral envelope proteins are formed. The virions, the subviral particles and HBeAg are secreted from the cell. [101, 109] Additionally, virions with empty capsids, lacking the HBV genome, are trafficked and released. It is hypothesized that the genome exhibits a blocking signal that ensures the maturation of the virion before release, while a capsid lacking the genome has no mechanism to block the secretion [142]. While this seems to be a disadvantage for the virus, because multiple types of non-infectious particles use the HBV proteins essential for the Dane particle, the subviral particles and empty virions might serve as distractors for the immune system and thereby ensure the viral spread.

### 1.2.3 The Disease

#### 1.2.3.1 Epidemiology & Transmission

257 million people were infected with HBV in 2017 with about 887 000 deaths being attributed to hepatitis B and its caused complications like cirrhosis and liver cancer (hepatocellular carcinoma) every year [1].

Prevalence of hepatitis B is very heterogenous throughout different regions of the world (Figure 4). High endemic regions with more than 50 % of the population being anti-HBc positive are Asia, Sub-Saharan Africa, the South Pacific, South America and the Middle East. Regions with medium prevalence rates of 10 – 50 % of the population being anti-HBc positive are East Europe and the Mediterranean Basin [1].

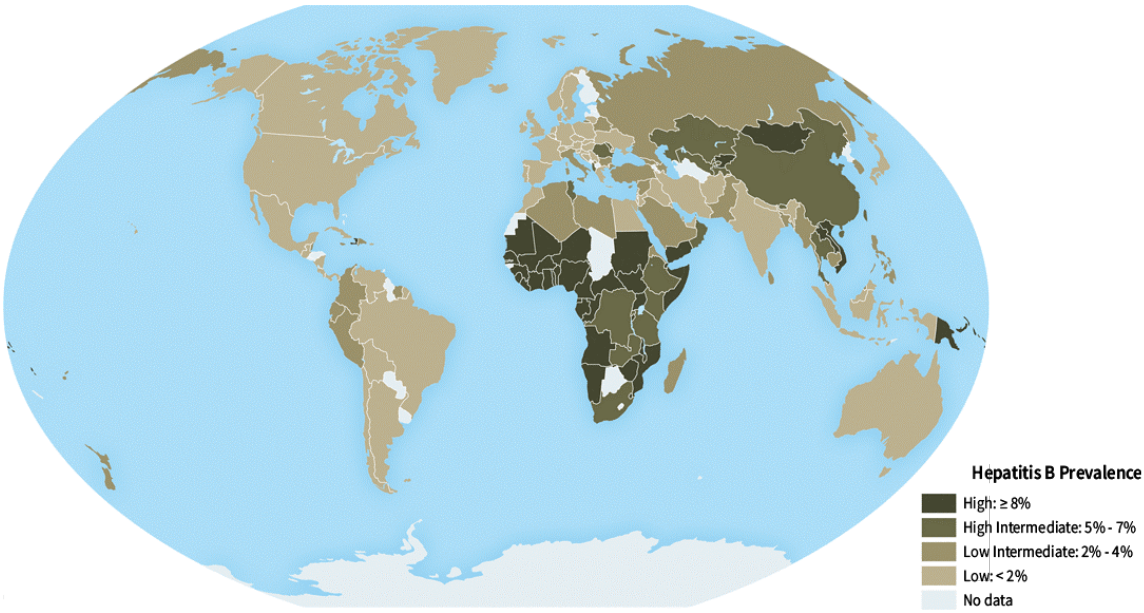


Figure 4: **Geographical hepatitis B prevalence** [143] based on [144]. Incidence of hepatitis B is shown around the globe and indicates higher prevalence in medium to dark brown colors with the highest incidence in Sub-Saharan Africa and East Asia.

Since hepatitis B is mainly contracted through blood or other body fluids of infected patients, the reuse of needles and syringes, which is present in drug abuse or uninformed health care settings, but also the share of razors, tattooing and even medical, surgical or dental procedures bring people at risk. Instruments have to be properly sterilized, because wiping them off is not sufficient and HBV particles are stable outside of a body for up to

7 days. Another big contributor to the worldwide prevalence of hepatitis B is perinatal transmission – the transmission from mother to child during birth. [1, 145]

High risk groups to contract HBV infection are people with non-HBV related elevated liver inflammation markers, liver fibrosis, liver cirrhosis or HCC, but also migrants from high endemic countries, biological children of HBsAg positive mothers, family members of or people in close contact to infected, medical care personal, pregnants, patients in psychiatric wards and institutions of care, dialysis patients, transplant recipients before and after transplantation, blood-/tissue-/sperm- and organ donors, patients before and during immunosuppressive therapy or chemotherapy, HIV infected, HCV infected, homosexual men, persons with regularly changing sex partners e.g. but not only restricted to sex workers, prisoners, current and former intravenous drug abusers. [1, 143]

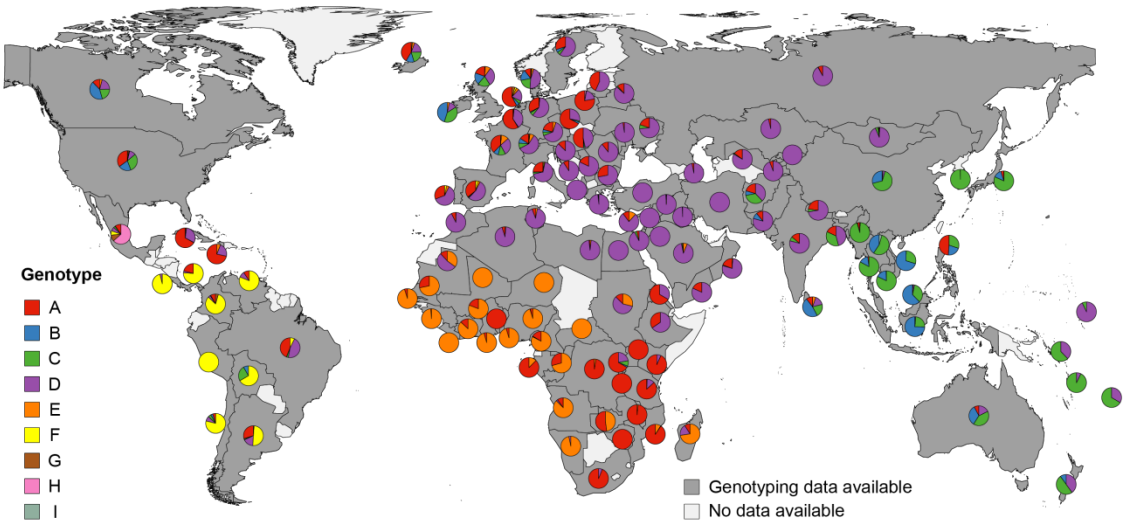


Figure 5: **Distribution of hepatitis B genotypes by country** [146]. The distribution of HBV genotypes in the respective country is indicated by pies. Color coding relates to the genotype as shown in the legend on the left.

Currently nine distinct HBV genotypes are differentiated (with genotype J being controversial), which are defined as strains with genome sequence variation of at least 8 %. Strains with lesser variance (with a difference between 4 – 8 %) can be defined as subgenotypes of which over 30 types are described [146].

90 % of all HBV infected are infected with genotypes B, C and D, while genotype A is prominent also in Africa, America, Europe and India (Figure 5). Currently no evidence is found to show a strong impact on disease outcome of infection with a distinct genotype. Additionally, genotyping is not prominently performed in the clinic since treatment is not changed according to the genotype, but the disease stage and viral parameters (like HBV DNA load, HBsAg levels, HBeAg status) as stated by the DGVS Leitlinien [130] and also the AASLD (American Association for the Study of Liver Diseases) recommendations [147]. [1, 146]

Different serotypes, which are defined as groups of virus strains with sets of antigens that have a distinct sequence, are recognized and bound by different antibodies. HBV has four major serotypes differentiated by the epitopes on the surface antigen. All four share the disulfide bond containing a determinant of HBsAg (aa 99 – 168), but combine distinct mutually exclusive determinants d/y [148] and w/r [149]. This results in the four major serotypes adw, adr, ayw and ayr. Additionally, the determinants can be differentiated into subdeterminants like w1 – w4. This increases the virus' variance greatly. There is also a q-determinant that is found on some serotypes increasing the possibilities even more. [150]

#### 1.2.3.2 Pathogenesis

Hepatitis B can manifest in a broad range of heterogeneous forms. The incubation time is 30 – 180 days, averaging at 75 days. Diagnosis via detection of the infection is possible after 30 – 60 days p.i. After initial HBV diagnosis, HBsAg positive persons should be tested for liver function and therapy options are supposed to be evaluated. Vaccination against hepatitis A is indicated. [130]

Most (~60 %) infected individuals have an asymptomatic hepatitis B and the infection is only detected in an unspecific check-up or only when late-term complications like cirrhosis or cancer appear [151, 152]. In adults, 95 % of infected are clearing the infection, whereas

80 – 90 % of infected infants develop chronic hepatitis B. This is similar to immunocompromised patients, where 30 – 90 % also develop persistent infections. [1]

The major hallmarks of acute hepatitis B is a duration of up to 6 months with loss of appetite, muscle or joint or stomach pain and fever, but also typical signs of liver inflammation like jaundice or bloated stomachs and GI tract problems. Common symptoms of hepatitis B patients are jaundice, dark urine, abdominal pain, nausea, vomiting and extreme fatigue. [153, 154]

The clinical picture is unspecific for hepatitis B and can therefore be confused with other viral hepatitides (besides hepatitis B, there are hepatitis A, hepatitis C, hepatitis D, hepatitis E, whereas also other viruses like CMV (cytomegalovirus), EBV (Epstein–Barr virus), YFV (yellow fever virus) and many more can cause liver inflammation). Therefore, serological analysis has to be conducted to diagnose the infection. [155]

In the laboratory, acute hepatitis B is identified as HBsAg positive, as well as anti-HBc IgM positive (Figure 6). In the beginning, the patients are also HBeAg positive indicating high viral replication and high viral blood titers making the blood very infectious. [156, 157] While patients with acute hepatitis B do not receive any antiviral treatment, an evaluation of anti-HBs antibody levels is repeated every 3 – 12 months until the titer reaches > 10 IU/L. [130]

Fulminant hepatitis is a rare, but severe disease state with fast and strong liver inflammation during the first 8 weeks of infection that leads to necrosis-induced reduced liver function and coma. Without transplantation up to 80 % of patients die, but successful transplantation usually improves the life expectancy and leads to a good long-term prognosis. [158, 159]

Chronic hepatitis B is characterized by the persistence of the virus infection and sustained levels of HBsAg (Figure 6). Due to the nature of persistent infections, patients with hepatitis B are also showing immunotolerant phases. Patients with chronic hepatitis B are more likely to progress to a worse disease stage if the viral load in the serum is > 2 000 IU/mL HBV-DNA, which equates to 10 000 copies of DNA/mL. [130] There are cases of spontaneously cleared chronic hepatitis B under antiviral therapy, but also in untreated patients [161, 162].

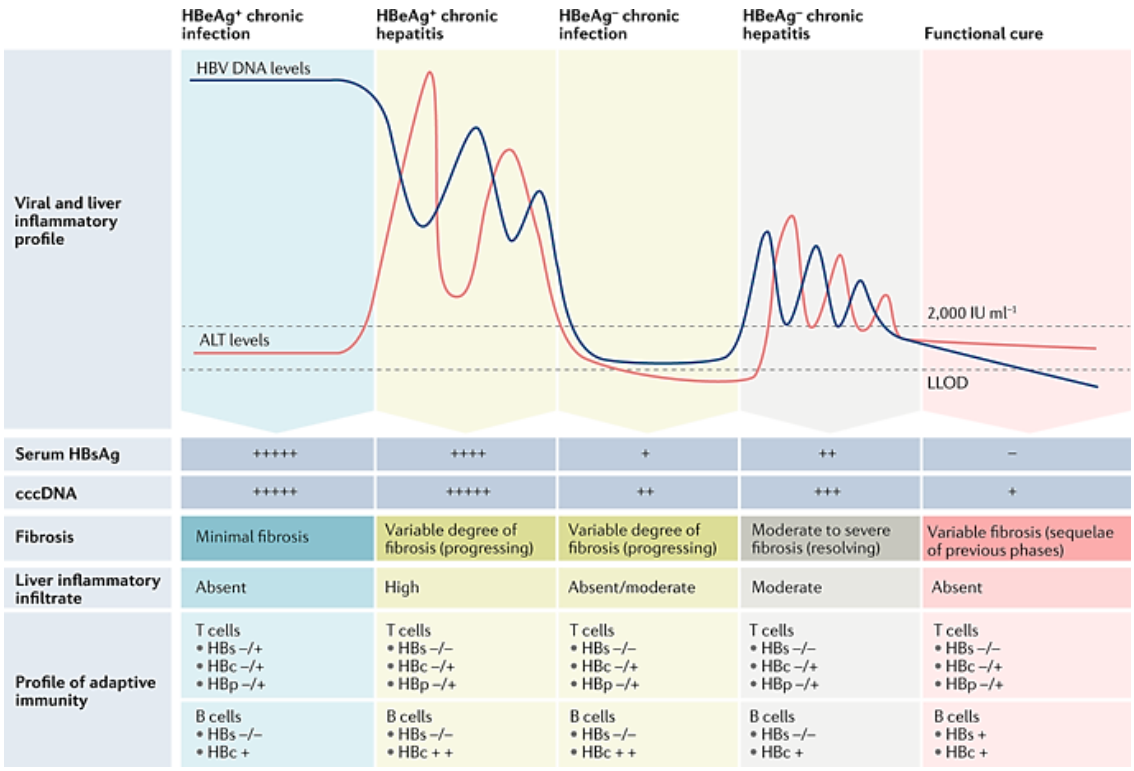


Figure 6: **Natural history of hepatitis B** [160]. Different stages of the HBV infection and development of hepatitis B are differentiated and indicated above. Typical relation of HBV DNA and ALT course are shown, as well as serum HBsAg and cccDNA levels. Fibrosis staging and the status of liver inflammatory infiltrates are given. Additionally, the profile of the adaptive immune system regarding T and B cells specific for HBV antigens are annotated.

Special cases of chronic hepatitis B are the anti-HBc-only and the occult infection. The first type is defined by HBsAg negative, anti-HBc positive and anti-HBs negative or < 10 IU/L with a low viremia (HBV DNA < 20 IU/mL). The prevalence in Germany is at 1.4 – 2.2 % and found especially in patients 65 years of age or older and with HCV (Hepatitis C virus) co-infection. Patients with anti-HBc-only status do not show liver inflammation, but can be HBV transmitters or even undergo hepatitis B activation upon immunosuppression. [130, 163, 164] In the second case, patients with occult HBV infection showing no HBsAg, but are HBV DNA positive (< 200 IU/mL, which equals to 1 000 copies of DNA/mL). Persistent, occult HBV infection is found in patients with HCV or HIV-1 co-infection, cryptogenic liver cirrhosis or HCC. Transient, occult HBV infection is sometimes found in patients who were immunized, but whose titers decreased below 100 IU/L. Though asymptomatic, patients with occult HBV infection can still transmit the disease or suffer from activation of hepatitis B due to immunosuppression. [130, 164, 165]

HBV infections are in general viewed as non-cytopathic, although the infection certainly has a detrimental effect on cell viability/vitality [166]. One possible cellular cytopathic effect of HBV infections in the form of so-called ground glass hepatocytes was found, which is identified as an aggregation of HBV envelope protein [167]. This could be a source of HBV replication-related inflammation, which in turn induces tissue repair. Repair includes the deposition of extracellular matrix that causes progressive liver fibrosis [168]. Also, the HBx protein might play a role in fibrogenesis and is also thought to be an oncogene [169-172].

Even with normal liver enzyme levels and controlled HBV DNA loads, patients often present with stage 2 inflammation and fibrosis in the case of a biopsy [173]. The infection, activation of the JAK/STAT pathway and cellular presentation of viral proteins drive inflammation and fibrogenesis [174-176]. Fibrosis, which can regress upon HBV treatment, is characterized by the proliferation of myofibroblasts after apoptosis of hepatocytes and the thereby resulting pathological scarring [177-179]. The most severe scarring stage of fibrosis is defined as cirrhosis with a functional reduction with portal hypertension and, what is defined as, end-stage liver disease [180, 181]. Reduced survival rates (5-year survival rate reduced from 97 % to 55 %) and increased incidence of hepatocellular carcinoma (HCC) are hallmarks of patients with cirrhosis [182]. The only curative option is liver transplantation. In addition to pathological remodeling of the liver, which leads to serious loss-of-function of one of the body's most important organs, the development of cancer is another life-threatening result of ongoing liver infection [183, 184].

The most common liver cancer is HCC, which is hard to remove completely with surgery (only 10 – 20 % of resections) leading to death within 3 – 6 months after diagnosis in most patients. Hepatocellular carcinoma is caused by HBV infection in 55 – 60 % of cases. Inversely, 10 – 17 % of annual hepatocellular carcinoma cases are caused by hepatitis B related liver cirrhosis. HCC can be treated with surgical resection, liver transplantation, chemo- or radio- or immunotherapy. [185]

Cure of hepatitis B is currently defined as the clearance of all viral molecules and normalization of clinical parameters. While some patients show a clearance of the disease, most persistently infected patients do not achieve cure under antiviral therapy even though cells with HBV cccDNA or HBV-genome integrates are found in patients who do not show any symptoms. This cannot be viewed as a cure due to the fact that a suppressed infection

can be reactivated in case of strong immunosuppression or chemotherapy. Therefore, the term of functional cure was established to define a state of patients without complications related to hepatitis that underwent HBsAg seroconversion (meaning the loss of HBsAg with the emergence of anti-HBs antibodies). Complications can still be observed in these seroconverted patients since a reactivation of the HBV infection is reported in lymphoma patients or under immunosuppressive treatment e.g. with corticosteroids or Rapamycin because the important control by B cells is missing. [186-193]

#### 1.2.3.3 Immune Responses to Hepatitis B

Upon HBV infection, CD4 and CD8 T cells specific to all HBV proteins are primed [194-197]. Nevertheless, HBV-specific T-cell frequencies are low [198] as they are possibly deleted due to Bim-mediated apoptosis [199]. T cells specific to HBV core were more prominent than to the envelope and polymerase proteins with the HBV X protein challenging the least T cells [200]. HBV-specific T cells only start to circulate in the periphery 6 – 8 weeks after infection [201].

HBV-specific T cells mainly home to the liver and can be detected by staining in diseased hepatitis B patients [202-204]. This is promoted by the slow blood flow in the liver [205], as well as the circumstance that T cells can not only adhere to epithelial cells in the venules, but also to LSEC (liver sinusoidal endothelial cells) in the sinusoids, which increases the number of lymphocytes in the liver substantially [206]. While T cells of all specificity are found in the liver, HBV core-specific and HBV env-specific CD4 T cells are most prominent in persistent HBV infection, while their function is not clear [207, 208]. In the context of hepatitis B, T cells infiltrate the liver before the inflammatory cascade triggers liver damage [209]. The inflammatory microenvironment in the liver can be promoted by hyper-activation of T cells e.g. due to the influx of LPS (caused by a permissive mucosal barrier or fibrosis) [210-213].

It was shown before that patients with resolved hepatitis B, as well as vaccinated individuals, have a strong memory T-cell population [214-216]. Upon vaccination with the recombinant HBsAg-based vaccine, not only high anti-HBs antibody levels, but also multi-specific and polyfunctional T cells are regarded as correlates of protection [197, 217, 218].

Polyfunctional T cells, secreting multiple cytokines in parallel, are reported to be present in acute HBV infection, but strongly decreased in chronic hepatitis B [219, 220].

Mainly, IFN-γ is secreted by HBV-specific T cells in the liver, which is mirrored by peripheral HBV-specific T cells [221, 222]. While HBV-specific T cells also secrete TNF and IL-2, IFN-γ secretion seems to be the main cytokine effect of HBV-specific CD8 T cells [223]. Antiviral $T_h1$ cytokines can induce fever, inflammation and inhibit viral replication. This was also specifically shown in the context of HBV, where TNF and IFN-γ can induce hepatocytes to produce IL-32, which in turn blocks HBV transcription and replication by downregulation of transcription factors [224]. It was shown that CD8 T-cell activation and liver-residence is dependent on platelet function and possibly on CD44 expression [225, 226]. In the liver, early expression of interferon stimulated genes (ISGs) is observed in hepatocytes (STAT1), while later expression is taken over by Kupffer cells [227-231].

Kupffer cells (Liver macrophages) secrete IL-6 and also proinflammatory cytokines in an NF-κB-dependent manner upon recognition of HBV. In hepatocytes, IL-6 cannot only control HBV transcription and replication, but also inhibits the essential transcription factors HNF-1α and HNF-4α and induces acute phase reactants. [132, 232]

It is well known for hepatitis B that the clearance of the acute HBV infection is associated with functional CD4 and CD8 T cells, while the infection becomes persistent after a failed and/or scarce T-cell response [233]. During acute HBV infection, HBV-specific T cells are partially depleted by NK cells with high TRAIL expression [234]. Although T-cell dysfunction is quite commonly described in HBV infection, it is only observed after prolonged infection [235, 236]. It is speculated that mainly HBV polymerase-specific T cells are dysfunctional while HBV env-specific T cells seem to be rather depleted [237]. It was shown before that persistent HBV infection induces Treg, which are central to the dysfunction of HBV-specific T-cell responses [238, 239]. Aside from T-cell dysfunction caused by regulatory immune cells, suppressive cytokines, low expression of costimulatory molecules and high expression of inhibitory receptors or the immunotolerogenic liver microenvironment (e.g. arginase, IDO), T-cell exhaustion is also reported for chronic hepatitis B [240]. HBV pol-specific T cells were found to be more frequently exhausted than HBV core-specific T cells [237].

B cells are lymphocytes releasing a wide variety of antibodies upon activation. Although HBV is deemed a stealth virus, because it can avoid detection and antagonization by the immune system [161, 241-243], HBV-specific antibodies against core and the envelope protein are induced upon infection and can lead to resolution of the infection. The main HBV epitope loop is called the 'a' determinant that is found within the surface antigen at the amino acid sequence position 124 – 147. Naturally, neutralizing antibodies regularly target the 'a' epitope [244], which physiologically interacts with the HBV entry receptor NTCP [133], or the heparane sulfate binding domain in the HBs antigenic loop [245]. This is also mimicked by prophylactic vaccination with recombinant HBsAg inducing anti-HBs (but no HBc) antibodies [246]. Patients who cleared an HBV infection (independent of acute or persistent infection) are therefore identified as HBsAg negative, anti-HBs positive and anti-HBc positive [130, 247]. The latter antibodies are correlated with the extend of liver damage [248]. Diagnostic of HBV infected patients is performed by analyzing HBsAg, anti-HBc, HBeAg, anti-HBe and also by quantifying the HBV DNA load. High titers of anti-HBs show a resolved infection or a successful vaccination (if HBc antibodies are not present). [130, 247]

#### 1.2.3.4 Prevention and Treatment

Since 1981 a vaccine against hepatitis B is available. The idea was first developed in the 1970s by Maurice Hilleman, who collected blood from gay men and drug users who had high incidences of hepatitis B and isolated the HBsAg to use it in the first trials [98]. This first HBV vaccine was discontinued in 1986 and replaced by a recombinant vaccine containing yeast-produced antigen by Pablo Valenzuela, which stimulates naive CD4 T cells with HBsAg [99]. Primed CD4 T cells are then able to prime and activate B cells via TCR/MHCII and CD40L/CD40 interaction to produce anti-HBs antibodies and induce central memory T-cell responses [249].

The current vaccine is based on genotype A (most prevalent in Europe and North America) and achieves a strong immune response that lasts for ten years [146, 250]. Vaccination reaches coverage of 84 % worldwide and shows a success rate of 95 % to prevent infection, the progression to chronicity and HCC [1, 251-253]. In Germany, it is recommended to vaccinate all anti-HBc negative children and adults against HBV [254].

Current vaccine trials target the varying effectivity to different genotypes or the application of DNA-based vaccines [255, 256]. So far, therapeutic vaccination is not available.

Current treatment of hepatitis B is mainly consisting of nucleotide and nucleoside inhibitors with the option of including peg-IFN-α (Figure 7). Nucleoside and nucleotide analogues inhibit the reverse transcription of HBV and are therefore called nucleoside reverse transcriptase inhibitors (NRTI), while peg-IFN-α stimulates an immune response to fight the infection [130]. It was shown that IFN-α treatment reduces cccDNA, HBV RNA intermediates and HBV antigens [257]. This is done by inducing APOBECs as well as interferon-stimulated genes (ISGs), currently attributed to ISG20 [258-260]. It also leads to smc6 reappearance indicating a suppression of HBx expression [261, 262].

Patients with acute hepatitis B are not treated, but medication for relieve of symptoms can be prescribed. Treatment of persistently HBV-infected patients depends on the state of HBV antigen and DNA loads. Therapy success with IFN-α or NRTI can be observed as a continuous drop in HBsAg [130]. Tenofovir also allows a safe treatment of pregnant women and reduces the perinatal transmission from 13.5 % to 0 % [263].

State of the art medications are IFN-α/pegylated IFN-α (approved in the EU since 2000/2002), the nucleoside analogues Lamivudine (Cytidine analogue; approved in the US since 1995, in the EU since 1996 for HIV-1 infections and 1999 for Hepatitis B), Entecavir (Guanosine; approved in the EU since 2006), Clevudine (Thymidine; approved in South Korea since 2006), Telbivudine (Thymidine; approved in the US since 2006, in the EU since 2007) and nucleotide analogues Tenofovir (Adenine; approved in the US since 2001, in the EU since 2002 for HIV-1 infections and Hepatitis B) and Adefovir (Adenine; approved in the US since 2002, in the EU since 2003). [130, 143]

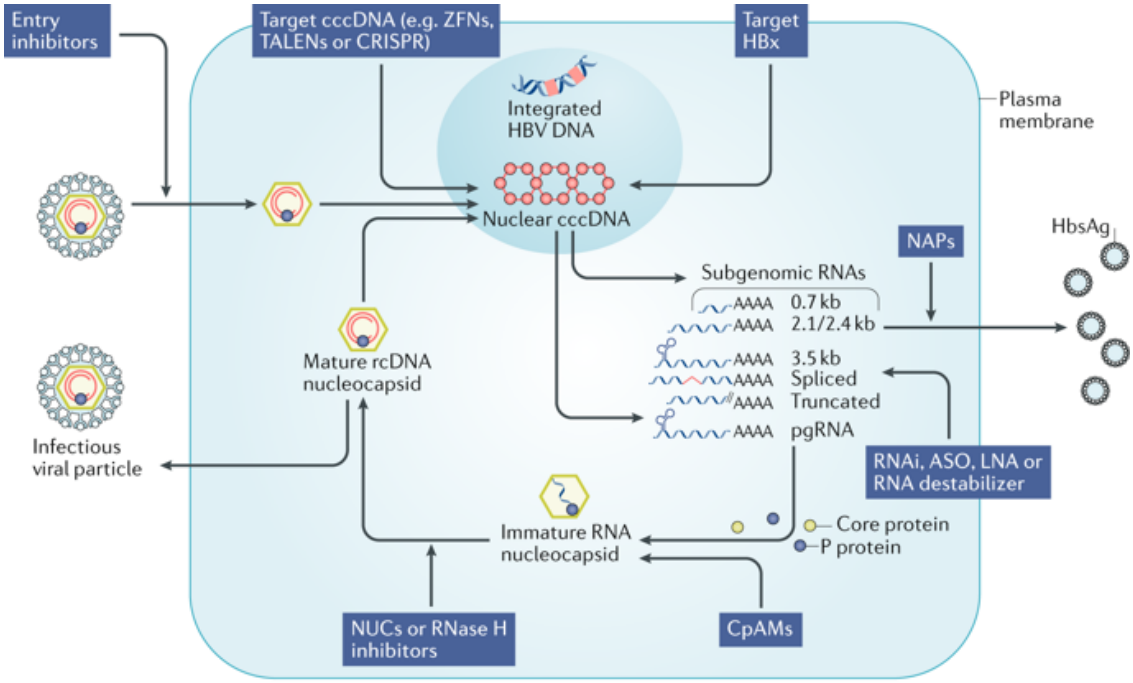


Figure 7: **Options for the treatment of hepatitis B** [160]. Treatment approaches for HBV infections are depicted, indicating their targets in the viral life cycle. Main options are to target the HBV entry, the persistent cccDNA or the HBx protein, which plays a role in transcription of the viral genome. The subgenomic RNAs, or the release of HBsAg, the capsid assembly or finally the reverse transcriptase (maturation step) can be inhibited.

After the successful development and deployment of the direct acting antiviral (DAA) Sofosbuvir against Hepatitis C in 2013 reaching clearance of this liver disease of up to 97 % [264], more focus of research and clinicians alike came to finding successes in hepatitis B treatments. Initiatives for new treatments were called upon by organizations like the newly founded ICE-HBV (International Coalition to Eliminate HBV), the Hepatitis B Foundation, the World Hepatitis Alliance and the WHO. [192, 265]

New treatment options target different parts of the HBV life cycle (Figure 7). One option is to inhibit the entry of virions to the host cell by inhibitors like Myrcludex [266, 267], which is a peptide antagonistic to the HBV entry receptor NTCP [133]. It was already shown in humanized mice that Myrcludex inhibits rebound, intrahepatic spread and cccDNA accumulation [268]. Several clinical trials are evaluating the effect [269-271].

The application of siRNA aims at reducing the translation of viral proteins and thereby decreasing viral particles and the secretion of HBV antigens. A promising new approach is the use of so-called CpAM, inducing the malassembly of virions by intercalating in the grove

between the capsid proteins [272, 273]. The capsid assembly modulator NVR 3-778 is in clinical trial stage [274].

Immunomodulatory treatments are discussed for the usage against hepatitis B or HIV for a long time [275, 276]. Multiple options like TCR or S-CAR therapy [277-280], as well as therapeutic vaccination [281-283], are tested to re-induce the dysregulated HBV-specific immunity or functional T cells [284, 285].

Aside from therapeutic vaccination, also checkpoint inhibition with anti PD-1 therapy can restore HBV-specific immunity as it is prominently performed in cancer patients [286]. It was shown that checkpoint inhibition is in some tumors restricted by RIG-I inactivation, which implies that a treatment with RIG-I activators could therefore enable the effect of checkpoint inhibition [287]. RIG-I and TLR agonists are nevertheless already in clinical studies to activate the innate immune response in hepatocytes or liver-resident immune cells [288-291].

## 1.3 HIV/AIDS

### 1.3.1 History of HIV/AIDS and major research breakthroughs

The HIV/AIDS pandemic is one of the greatest burdens of humankind. After its discovery in the 1980s, the disease spread rapidly around the world, becoming an ongoing pandemic and is now the no 1 cause of death among women aged 15 – 49 [292]. Françoise Barré-Sinoussi and Luc Montagnier discovered the human immunodeficiency virus (HIV) to be the source of the Acquired Immune Deficiency Syndrome (AIDS) in 1983 [293]. While the HIV infection has devastating effects on the health and life expectancy, it also means imminent discrimination for the patients and lowers their quality of life exceptionally.

### 1.3.2 Human Immunodeficiency Virus

#### 1.3.2.1 Phylogeny, genome and structure

The human immunodeficiency virus belongs to the family of Retroviridae and the genus of Lentivirus. Another main retrovirus is the human T-lymphotropic Virus 1 (HTLV-1) (also known as human T-cell leukemia virus 1). HIV is part of group VI in the Baltimore scheme containing viruses replicating their (+)ssRNA genome dependent on reverse transcription [294]. HIV is subdivided in HIV-1, which is the most prevalent type, and HIV-2. [101]

HIV infects mainly CD4 T cells [4, 7] and macrophages and monocytes [8], but also microglia [295] and dendritic cells [296, 297]. HIV strains can be distinguished by their tropism to the co-receptors CCR5 or CXCR4 into M-tropic (R5) or T-tropic (X4) strains, respectively. During sexual transmission, HIV firstly infects mucosal dendritic cells via either CD4 and CCR5 or DC-SIGN. Infected DCs connecting to T cells for antigen presentation might be responsible for the transmission of HIV to the T-cell population. In the acute phase of the infection, $CCR5^+$ CD4 T cells are infected, which are mostly mucosal CD4 T cells. As most mucosal CD4 T cells are found in the intestine, the primary infection resolves in these organs. Consecutively, HIV spreads via cell-to-cell spread via the virological synapse to other T cells [298] or during antigen-presentation from macrophages or dendritic cells to T cells [299]. Alternatively, HIV virions can also be distributed via cell-free spread [300].

Aside from immune cells, HIV can also infect glia cells like astrocytes and thereby affect the brain, where it also develops reservoirs [295, 301].

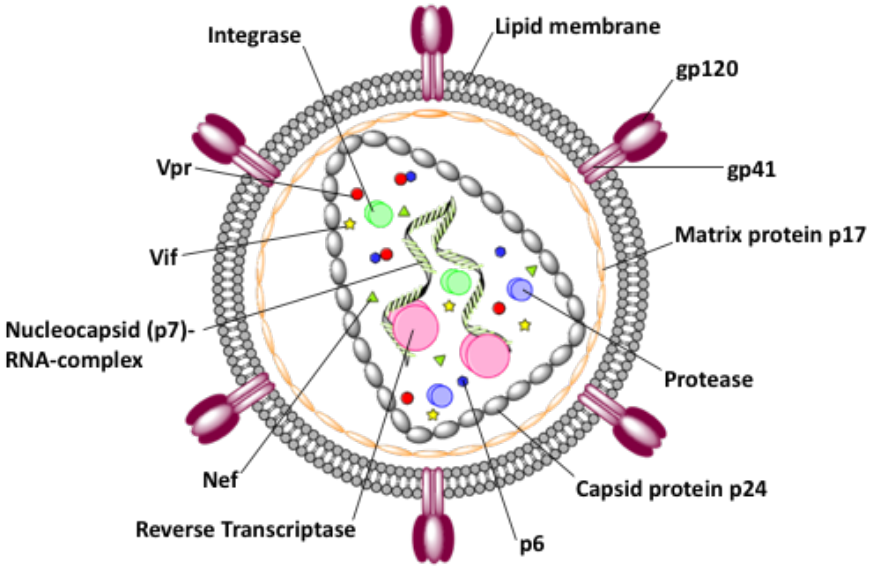


Figure 8: **Human immunodeficiency virus particle** [302]. Virions of HIV are enveloped by a lipid bilayer containing host cell membrane proteins and the viral proteins gp120 and gp41 of the glycoproteincomplex. The lipid membrane is seamed with the matrix protein p17. The capsid (p24) surrounds the two copies of RNA genome bound to the nucleocapsid p7 and the reverse transcriptase, the integrase, the protease, p6 and Nef, Vpr and Vif.

HIV particles are spherical with a diameter of 120 nm (Figure 8) [303]. The env glycoproteins are found on the lipid membrane [304]. Its inner layer is rimed with the matrix protein. The inside contains a conical capsid and the protease [305]. Inside the capsid, two copies of the ssRNA genome are bound to the nucleocapsid protein and associated with the reverse transcriptase [306], while the integrase, vif, nef and vpr proteins lie unbound [307-309]. The two genome copies provide a backup function, as well as enabling recombination and are used in strand switches during reverse transcription. The genome consists of nine genes encoding up to 19 proteins. At the 5' end of the genome, the LTR (long-terminal repeat) promotor is encoded, followed by the psi element, which is central to genome packaging upon recognition of the gag and rev proteins. The *gag* gene encodes the gag polyprotein consisting of the matrix protein (p17), the capsid (p24), a spacer peptide, the nucleocapsid, another spacer peptide and the p6 protein, which initiates budding. Due to the SLIP element, a frameshift in the *gag* gene produces the gag-pol polyprotein (p160). Although the polyprotein includes the sequences for all the gag-proteins, it only encodes for the

nucleoprotein and additionally is a template for the transframe protein, another p6 pol protein, the protease, the reverse transcriptase, the RNaseH subunit and the integrase. The *env* gene codes for the gp160 protein, which is cleaved by the host cell's protease furin to the gp120 and gp41 envelope proteins. [101, 310]

The accessory proteins vif, vpr, tat, rev and vpu are encoded between and overlapping to the pol and the env polyproteins, followed by the final accessory protein nef. They altogether enable the successful replication. Vif is an antagonist of the ISG APOBEC3G. Vpr inhibits the cell cycle and cell division. The two tat genes encode the transactivator of transcription that binds to the TAR binding element (which also inhibits apoptosis [311]) of the LTR promotor. Rev plays an important role in shuttling viral RNAs from the nucleus and regulating RNA splicing (and thereby the HIV protein production) by binding of the Rev-responsive element. The vpu protein regulates the release of viral particles. Nef is an immune evasion protein that downregulates CD4 and MHC I and II. It is followed by the 3' LTR. [101, 310]

#### 1.3.2.2 HIV Life Cycle

HIV-1 approximates to target cells via binding to heparane sulfates, glycosaminoglycans, followed by attachment and interaction of the gp120 proteins to the CD4 receptor and the CXCR4 or CCR5 co-receptors [312, 313] (Figure 9). The lipid membrane fuses in a gp120-dependent manner to the cell membrane after penetration by gp41 and releases the capsid to the cytosol [314, 315]. The so-called reverse transcription complex is formed containing the viral RNA, RT and ribonuclease and the integrase, which is transported to the nucleus, while the (+)ssRNA is transcribed (3' to 5' with a tRNA primer) to DNA and eventually (by strand hybridization) to dsDNA [316, 317]. The RT complex is abolished, while the pre-integration complex (PIC) forms containing the viral DNA, integrase, vpr and the matrix protein. The PIC enters the nucleus in a vpr/matrix-mediated manner through the nuclear pore [318, 319]. In the nucleus, the dsDNA is integrated into the host cell's genome by the integrase as the so-called provirus [310]. Due to the integration, the HIV genes are flanked by the LTRs. As long as the host cell is not activated, the HIV replication is latent. Transcription factors like NF-κB are needed for transcription, but are not sufficient for an effective transcription as long as the viral accessory protein Tat is not present, and

translation indicating that the HIV life cycle is only productive in activated immune cells [320].

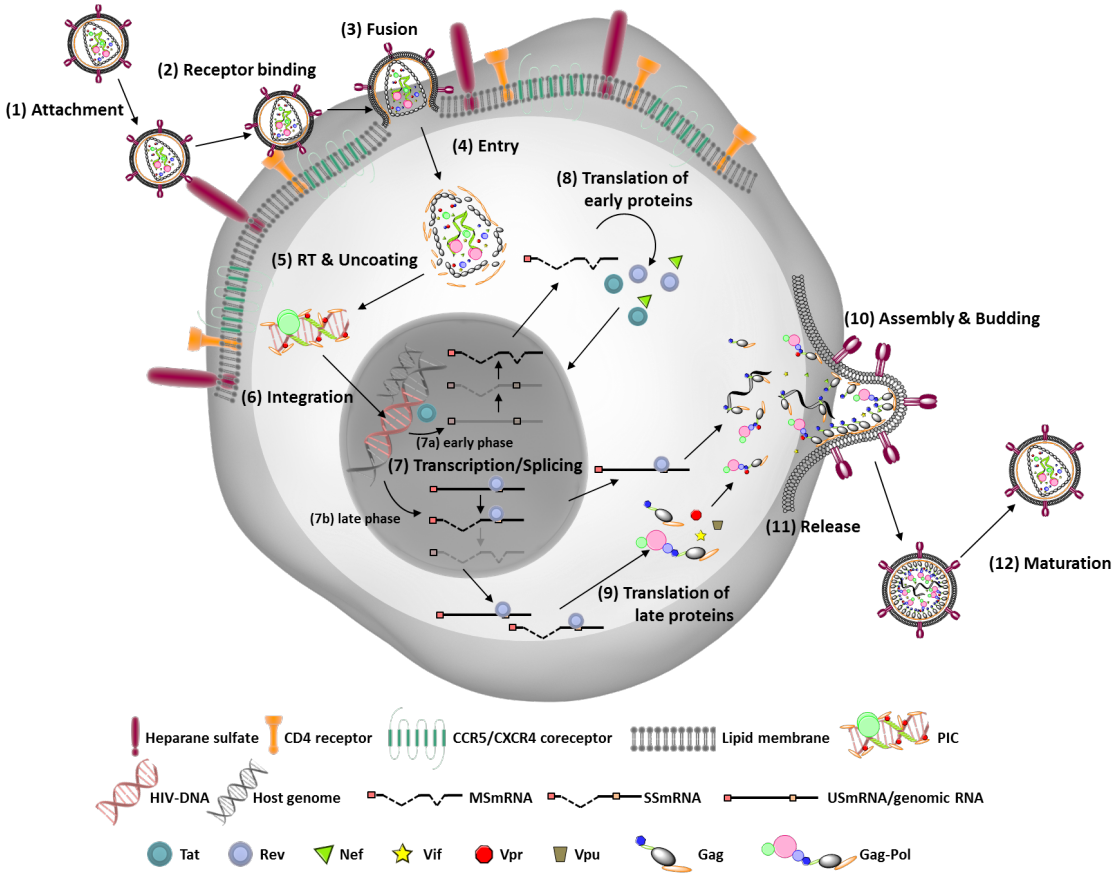


Figure 9: **Life cycle of the human immunodeficiency virus** [302]. (1) HIV virions attach to heparane sulfates and (2) bind the CD4 receptor and interact with the host cell upon secondary binding to the coreceptors CCR5 or CXCR4. (3) The lipid bilayer of the virus fuses with the cell membrane and (4) the capsid is released to the cytoplasm, (5) where the RNA genome is uncoated and reverse transcribed to DNA, which is shuttled to the nucleus. (6) The DNA is integrated to the host genome and (7) is transcribed to mRNA, which are partially spliced. (7a) First, the multiply spliced (MS) mRNA are transcribed and (8) the early proteins Nef, Rev and Tat are translated by host cell ribosomes. (7b) Then, newly produced Rev protein reenters the nucleus and leads to production of unspliced (US) and singly spliced (SS) mRNA, (9) which are translated to the late viral proteins. (10) The assembly takes place at the cell membrane, where the particles bud after tetherin cleavage. (11) The particles are released and (12) mature by gag protein cleavage outside of the cell.

In early phases of HIV gene expression, a full-length transcript, the so called pre-mRNA, is transcribed from the provirus. Extensive splicing of the pre-mRNA results in many different splice products encoding the HIV proteins Rev, Tat and Nef. Tat and Rev are shuttled back

to the nucleus and the viral expression is increased 100-fold by the binding of Tat to the TAR region [321, 322]. With a rising concentration of Rev in the nucleus, an increasing number of pre-mRNA transcripts are blocked from being spliced resulting in singly spliced and unspliced mRNAs enabling the production of the proteins Gag, Pol, Env, Vif, Vpr and Vpu [323, 324]. Viral proteins Env and Vpu are translated at the ER membrane and transported to the cell membrane via the ER/golgi route, whereas all other proteins are produced at free ribosomes in the cytoplasm [325]. Virion assembly takes place at the cellular membrane. Besides the gag- and gag-pol polyproteins, as well as two RNA genome copies, the accessory proteins vif vpr and nef are incorporated into the virus particles [308, 310]. After the budding, virions mature by the cleavage of the gag polyproteins by the HIV protease. [101, 310]

### 1.3.3 The Disease

HIV infection and acquired immunodeficiency syndrome are two facets of one disease now coined as HIV/AIDS. HIV infected patients develop AIDS, which destroys the immune system and results in death by opportunistic infections or cancer. [326, 327]

#### 1.3.3.1 Epidemiology & Transmission

According to the WHO, 37.9 million people are infected with the human immunodeficiency virus (HIV) worldwide. 770.000 people died from HIV infections in 2018 and 1.7 million people were newly infected. Overall, 32 million people are estimated to have died from the HIV pandemic so far. [292]

More than two thirds (about 68 %) of HIV infected live in the African region, especially Sub-Saharan Africa (Figure 10). The second highest prevalence is in South-East Asia with about 10 % of all HIV infected, followed by 9 % in the Americas, 7 % in Europe, 5 % in the Western pacific region and 1 % in the Eastern Mediterranean. [329]

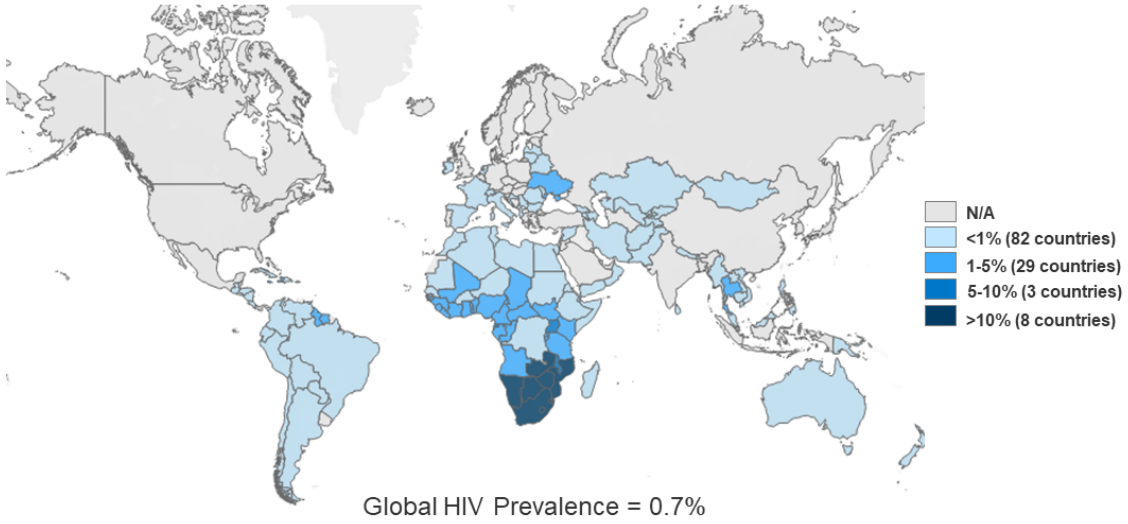


Figure 10: **Geographical prevalence of HIV infection** [328]. Incidence of HIV infections of adults (aged 15 – 49 years old) around the world is shown and is highest in Sub-Saharan Africa and especially Southern Africa indicated by middle and dark blue.

Regarding the WHO's HIV/AIDS targets, it was reported in 2018 that 79 % of people living with HIV know of their disease, 78 % of patients who knew their status (62 % of infected) received antiretroviral therapy and 86 % who received treatment (53 % of all infected) achieved sufficient suppression of the viral load to reduce the risk of infecting others. While this does not fulfill the set targets (90 %, 90 % and 90 % respectively), efforts to reduce the burden of HIV worked since the 2000s with decreases in HIV-related death (45 %) and new infections (37 %). [292, 330]

Main routes of infection with HIV are sexual intercourse and drug use, but also blood transfusion. Due to the successful suppression of the HIV load upon ART (antiretroviral therapy, formerly also known as (highly active-) HAART or (combination-) cART), HIV transmission via sex is ruled out if the infected partner has consistently undetectable viral loads (< 50 copies/mL) reducing the incidence to 0 % [331-334].

#### 1.3.3.2 Pathogenesis

HIV infection-mediated pyroptosis and apoptosis of immune cells lead to increasing failure of the immune system. While this becomes apparent when analyzing PBMC of the infected, clinically the immunodeficiency is more often found due to opportunistic co-infections. [327]

In the acute phase of HIV infections, about 50 % of patients develop flu-like symptoms e.g. fever, sore throat, rash, swollen lymphnodes, headaches or fatigue, but also GI tract symptoms like diarrhea and vomiting may occur. Rarely, patients present with neurological symptoms such as neuropathy and Guillain-Barré syndrome. [327]

After the acute phase, the infection transitions to a latent phase that runs for an average of 8 (3 – 20) years. 50 – 70 % of patients develop persistent lymphadenopathies for up to half a year. HIV-infected individuals do also develop liver disease like steato hepatitis [335].

Most subjects show decreased levels of CD4 T cells during the ongoing infection, eventually resulting in the development of AIDS [326]. Besides a small proportion of patients with long-term undetectable to low viral loads referred to as elite controllers [336-339], there are subjects whose CD4 levels do not decrease for a prolonged time called longterm non-progressors [340-342].

About 50 % of patients that contracted HIV develop the acquired immunodeficiency syndrome that is defined as CD4 T-cell counts/µL blood below 200 within 10 years. Initial symptoms are fever, swollen lymph nodes, chills and night sweats, fatigue and unintended weight loss. Additionally, patients with AIDS present with diarrhea and neurological, as well as psychological symptoms. Common opportunistic infections are pneumocystis pneumonia (40 %), HIV wasting syndrome (20 %), and esophageal candidiasis or other respiratory tract infections. Aside from secondary infections, many AIDS patients also develop viral-induced cancers. Kaposi's sarcoma (10 – 20 % of HIV-infected) and lymphoma (causing death in 16 % of AIDS patients) are associated with human herpesvirus 8 and are quite common in patients with AIDS. Cervical cancer and conjunctival cancer are also found commonly in HIV-infected. [326]

#### 1.3.3.3 Immune Response to HIV

Upon acute HIV infection, CD8 T cells attack and destroy infected cells resulting in the highest total loss of CD4 T cells during the natural history of the infection [343]. While this triggers the progressive immunodeficiency leading to AIDS and enabling opportunistic infections, chronic immune activation and inflammation are also reported. This underlines observations of clinically increased serum levels of pro-inflammatory cytokines [344]. One idea of this controversy describes that HIV introns induce innate immune activation of macrophages via MAVS signaling. This macrophage activation is observed to inhibit T-cell function, induce inhibitory receptors and T-cell exhaustion [10]. In turn, the bulk killing of mucosal CD4 T cells also leads to a loss of immune surveillance, but an increase in circulating LPS also further inducing immune activation especially in the GI tract and the liver [345].

Interferon induced genes are a main regulator of viral infections. While IFNs play a central role in inhibiting early HIV infection e.g. via APOBEC3G [346], Trim5a [347], Tetherin [348, 349] and SAMHD1 [350-352], interferons also contribute to the chronic immune activation, which drives HIV progression. Interferon induced transmembrane proteins (IFITM) inhibit not only the viral entry, but also the HIV protein synthesis by keeping viral mRNA from the translation machinery. This is in-turn counteracted by the HIV protein nef [353]. Nevertheless, HIV accessory proteins vif and vpu inhibit the effect of ISGs [354].

#### 1.3.3.4 Treatment and Prevention

Antiretroviral drugs are available since 1987 (Zidovudine/AZT) with approval by the FDA and the EMA [355, 356]. While treatment of HIV is effective by reducing the viral load and slowing the progression of the disease, even successful ART does not achieve cure. Due to HIV reservoirs that are not reached by the drugs, interruption of the therapy leads to re-emergence of the latent infection in cells with integrated provirus [357].

Two cases of cure from HIV are reported: Timothy Brown and Adam Castillejo. These were patients with leukemia or lymphoma, who received a bone-marrow transplant from HIV-resistant donors with the Δ32 mutation in the CCR5 gene. As this cannot be performed

for every HIV-infected patient, there is still no general cure available. These cases give the opportunity to determine a possible cause for HIV cure, which is discussed to depend on the graft versus host disease caused by the transplant. [358-360]

Antiretroviral therapy was first tested in a clinical trial in 1995 by the US national institute of allergy and infectious diseases in cooperation with the company Merck. Typically, two NRTI are combined with one NNRTI, a protease inhibitor or an integrase inhibitor [361, 362] (Figure 11).

The first drug class developed for the treatment of HIV was NRTI (Nucleoside/nucleotide analog reverse-transcriptase inhibitors) in 1987, which are still in use in every ART combination. Lamivudine, Abacavir, Emtricitabine, Entecavir and Tenofovir are examples of HIV-active NRTIs. These drugs are incorporated into the DNA and block the reverse transcription by chain termination. [363]

Protease inhibitors are available since 1995. There are multiple protease inhibitors on the market for example Ritonavir, Lopinavir or Darunavir. These drugs block the proteolytic cleavage of the gag and the pol polyprotein precursors and thereby inhibit the production and maturation of viral particles. [364]

The first HIV-active NNRTI (Non-nucleoside reverse-transcriptase inhibitors) was approved in 1996. NNRTIs bind non-competitively to the reverse transcriptase and induce a conformational change that inhibits the polymerase activity blocking the HIV replication. Drugs in this class are for example Efavirenz, Delavirdine, Rilpivirine and Doravirine. [365]

So far, only three entry inhibitors, fusion inhibitors and co-receptor inhibitors are approved for the treatment of HIV since 2003. These are Enfuvirtide (binding gp41), Maraviroc (binding CCR5) and Ibalizumab (binding CD4). [362]

The first integrase inhibitor Raltegravir was approved by the FDA in 2007. Only three other integrase inhibitors reached the market with Elvitegravir, Dolutegravir and Bictegravir. HIV-active integrase inhibitors block the integration of the provirus, which is conducted by the HIV integrase. [366, 367]

In addition to drugs that have direct antiviral function, two drugs are used as boosters of the pharmacokinetics of antiretroviral drugs. While Ritonavir is a protease inhibitor itself, it is now only used to inhibit the metabolism of protease inhibitors by the liver resulting in

higher and prolonged blood levels of HIV-active drugs. Cobicistat has a similar effect, but is not an antiretroviral drug itself. [368, 369]

ART can also be used to prevent the HIV infection as so-called PEP (post-exposure prophylaxis). The drugs have to be applied within 48 – 72 h after the possible infection [370, 371]. Additionally, HIV-active drugs can also be used as PrEP (Pre-Exposure Prophylaxis). A pre-emptive treatment that can be taken rather spontaneously before a possible infection event occurs. The treatment efficacy is very high, although the approval sparked controversy about the ethics of giving drugs to enable care-free sexual contact or to sex workers. [372, 373]

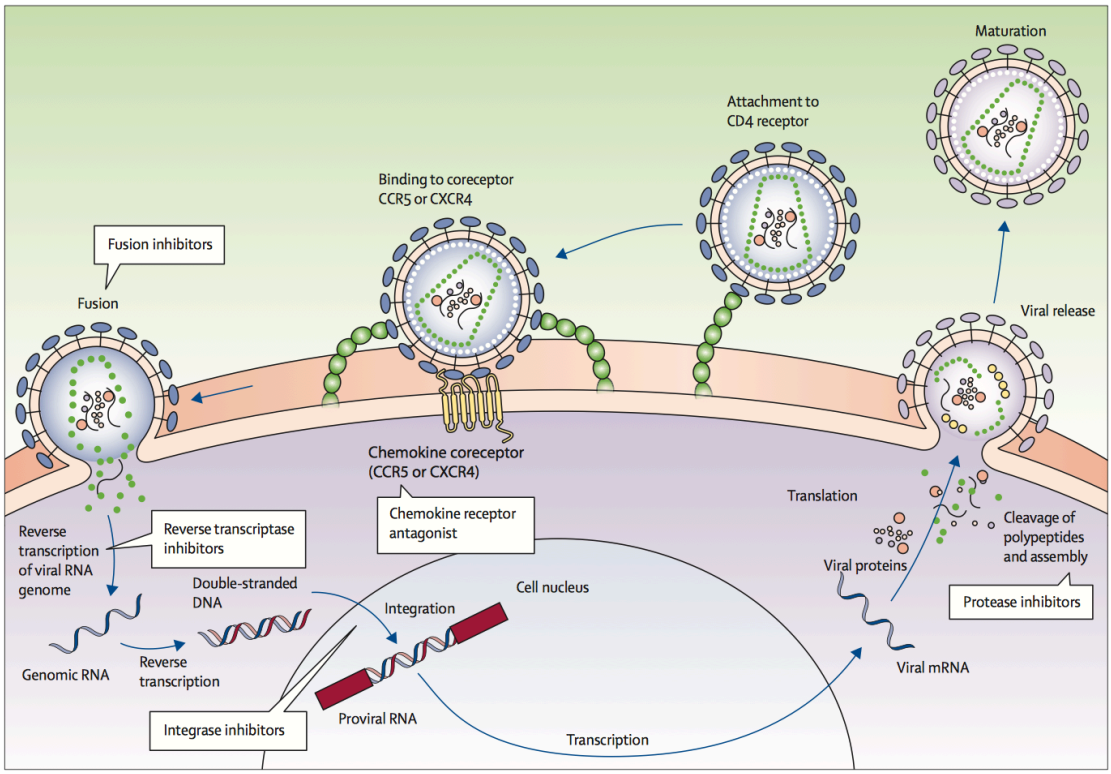


Figure 11: **Drug targets in the HIV life cycle** [374]. HIV infection can be blocked during the entry of the virion by chemokine receptor antagonists or fusion inhibitors and directly afterwards with reverse transcriptase inhibitors while the viral RNA is re-transcribed to dsDNA. When the dsDNA enters the nucleus and is integrated to the host's genome, the integration can be blocked with integrase inhibitors. After transcription and translation of viral proteins, protease inhibitors can downregulate the cleavage of polypeptides before the virions are assembled and released. Finally, the virions mature outside of the host cell.

Treatment options under development for HIV are broad. While new drugs of already used drug classes like NRTIs are in clinical trials, other mechanisms and targets are explored. Maturation inhibitors are a class of HIV drugs that block the final step of the gag protein processing. No drug of this class is approved so far and the development of multiple maturation inhibitors was discontinued [375, 376]. As more HIV-1 broadly neutralizing antibodies were discovered since 2006, they were tested in viremic individuals showing to be well-tolerated and reducing the viral titers [377]. They have advantages over the currently used HIV-active drugs as they are cheaper, have a longer half-live time and harbor the opportunity to induce the immune system-mediated killing of infected cells.

Besides researching new drug classes for keeping the HIV infection under control and slowing the progression [378, 379], the main targets of anti-HIV treatment development are latently infected cells and to clear viral reservoirs [380]. The Shock and Kill approach targets latently infected cells [381]. Latency reversing agents (LRAs) are used to reactivate the latent virus infection (shock) for the cell to be detected and destroyed by the immune system (kill). Latency reversing agents in trial are for example Histone-deacetylase inhibitors (HDACi), Histone methyltransferase (HMT) or DNA-methyltransferase inhibitors. These drugs use epigenetic targets to reverse the latency [382]. While this approach seemed to work *in vivo*, it was shown that the effect is only limited and might be inhibited by the anti-apoptotic capacities of LRAs [383, 384]. Nevertheless, other ideas are still under development do reverse latency. BAF inhibitors are under investigation to inhibit nucleosomal positioning and reverse the transcriptional repression of HIV [385]. As shown in preprints, FoxO1 inhibition is a potential novel LRA [386]. Immunomodulatory approaches are developed for the reversal of HIV latency. A study tested the combination of the IL-15 agonist N-803 with the depletion of CD8 T cells in macaques infected with SIV (simian immunodeficiency virus) under antiretroviral therapy reporting a persistent reactivation of HIV replication. Interestingly, a co-culture with CD8 T cells blocked this effect. [387]

The Hit & Cut approach uses the crispr/cas9 system to achieve cure of HIV/AIDS. The idea is that the guide RNA identifies and targets the HIV provirus and either induces mutations to inactive it or cut it out of the host's genome. [380, 388, 389]

## 1.4 HBV/HIV co-infection

HBV co-infection is most commonly described as an opportunistic infection of HIV-infected patients. The natural history of HBV/HIV co-infected patients is more complicated, as well as more fatal than that of both mono-infections. [1, 292, 390]

### 1.4.1 Epidemiology

Of patients with HIV-1 infection, 70 – 90 % are or were also infected with HBV, while 7.4 % have an active HBV infection. Conversely, of all HBV-infected individuals 1 % are also HIV co-infected. This makes up for 2.7 million HBV/HIV co-infected people. Regions that are strongly affected are Sub-Saharan Africa and Southeast Asia, both of which are also burdened by a high prevalence of hepatitis B and HIV/AIDS. HBV/HIV co-infection is nevertheless a global health threat. [1, 292, 391-393]

Contraction of HBV/HIV co-infection is similar to the single infections. Both, HBV infection and HIV/AIDS are transmitted via blood and sexual intercourse. A study on soldiers in Sudan revealed sexual contact to be a main risk factor for contracting HBV/HIV co-infection [394]. Risk groups for HBV/HIV co-infection are sex workers, men who have sex with men and people who inject drugs [395]. Also, perinatal transmission is a source of global HBV/HIV co-infection, but can be reduced by successful antiviral treatment [396-398].

### 1.4.2 Pathogenesis

The immune response determines if an acute HBV infection resolves or progresses to persistence [233]. An underlying HIV infection-induced immune suppression, especially regarding T-cell depletion or dysfunction [399], can impair the HBV resolution increasing progression to chronic hepatitis B strongly [400, 401]. The course of HBV infections is altered upon HIV co-infection [402-404] regarding an increased HBV replication and lower rates of HBeAg seroconversion [405, 406]. HIV-induced CD4 T-cell depletion can also result in the loss of the immunological control that limits HBV infection-induced liver damage [407, 408]. This is homologous to tuberculosis and HIV co-infection, where antigen-experienced

CD4 T cells are depleted to a higher degree than antigen-naïve T cells [409, 410]. Alternatively, a primary HBV infection induces chronic T-cell activation that enables HIV to have more target cells and even increase the replication of the virus and the progression of HIV/AIDS. This was shown as the risk of an HIV infection to progress to AIDS is increased in persistent HBV co-infection [411].

HBV/HIV co-infected patients have higher mortality and morbidity (though independent from the degree of HBV suppression) than HBV mono-infected individuals. Tenofovir-based antiretroviral therapy reduces the mortality and morbidity and improves the disease outcome and the life expectancy greatly [411-417]. Nevertheless, successful ART initiation is linked to immune reconstitution disease (IRD) defined by a transient worsening of the patient's condition, which is observed for various opportunistic infections and is also described in HBV/HIV coinfection [418-420]. In this setting, IRD manifests as ALT increases and/or hepatic flares, the so-called immune reconstitution inflammatory syndrome (IRIS), which is discussed to be linked to spontaneous HBs seroconversion [419, 421]. The HCC incidence is higher in HBV-infected, as well as HIV-infected individuals than in the general population. Nevertheless, data on the prevalence of HCC in HBV/HIV co-infection is not available [422-424]. In 10 % of HBV/HIV patients, the HBV DNA load does persist even under tenofovir-based therapy [425] and the genome undergoes mutations that are linked to active, but not latent replication [426]. HIV infection itself leads to steato-hepatitis [335], which complicates the liver disease in HBV/HIV co-infection. Nevertheless, HBsAg seroconversion rates are higher (up to 7 – 36 % vs. 0 – 7 %) in HBV/HIV co- versus mono-infected patients [427-431], but might not be long-lasting [432, 433].

Due to the positive effect of effective ART on the life expectancy in individuals with HIV/AIDS, secondary symptoms and long-term complications are becoming more prevalent. Clinicians are in need to develop and revise guidelines for the management of chronic opportunistic diseases like progressive hepatitis [411].

In HBV/HIV co-infection, liver pathogenesis does not arise from a co-infection of hepatocytes, but the interplay of the immune system. While HIV mainly infects immune and central nervous system cells, cells in the liver like liver stellate cells [434] and Kupffer cells [435] can be physiologically infected by HIV. By contrast, HIV infection of hepatocytes is only artificial [436] and experimental co-infection in single cells remains controversial [437-439]. However, HIV proteins can affect hepatocytes and might therefore directly interact with the HBV life cycle [2].

### 1.4.3 Treatment and Prevention

While there is an effective vaccine against hepatitis B (see 1.2.3.4), none is available for HIV/AIDS. Although all HIV patients should receive HBV vaccination, effectiveness of the anti-HBV vaccine is limited in HIV mono-infected patients regarding protectivity from HBV co-infection [130, 440, 441]. HIV patients with isolated anti-HBc (and no anti-HBs) antibodies develop good anti-HBV responses upon three- to four-dose HBV vaccination [442].

For the treatment of HBV/HIV co-infection, drugs like the NRTI tenofovir (alafenamide and disoproxil fumarate) are approved by the FDA and EMA. Alternatives are lamivudine and emtricitabine [130, 443]. NRTI like Tenofovir are working against both HIV-1 and HBV infections, since they are RT inhibitors and both the HBV and the HIV life cycles rely on reverse transcription. Additionally, both viral polymerases have sequence homologies especially in the RT region [444, 445]. Higher resistance mutation rates to NRTI lamivudine and controversially tenofovir treatment are reported for HBV/HIV co-infected patients, which could be linked to these homologies [425, 446-451].

To circumvent resistance development, it is now recommended to combine tenofovir with lamivudine or emtricitabine [452, 453]. An alternative combination therapy with Trizivir (Abacavir/Lamivudine/Zidovudine) plus tenofovir was evaluated in a small study. The combination could be advantageous since no NNRTI or PI are used and can therefore be retained for a possible therapy change in case of resistance development [454].

As immunomodulatory therapies are developed for hepatitis B, the checkpoint inhibition PD-1 blockade is also discussed as a new treatment option for HBV infection. Its effect was also evaluated in HBV/HIV co-infection and increased HBV-specific CD8 T-cell responses and improved the survival of HBV-specific CD8 T cells [455].

## 1.5 Aim of this Study

HBV/HIV co-infection burdens about 2.7 million people, who face life-long antiviral therapy, complications and adverse effects. While HBV-active ART prolongs the life span of HIV/AIDS patients with hepatitis B greatly, no cure is achieved. Progressive liver disease, caused by chronic hepatitis B, is associated with fibrosis, which evolves to cirrhosis and HCC. The underlying mechanism is mainly infection-related immunopathogenesis in the liver that induces scarring of the tissue and loss of function. Analyses of immunopathogenesis and the causative T-cell immunity in HBV/HIV co-infection are limited, so far. Results that were reported by the studies are contradictory and controversial. It was shown by Chang et al. in 2005 and Zhang et al. in 2013 that there were no significant differences in T-cell responses in patients with chronic hepatitis B or HBV/HIV co-infection [456, 457]. By contrast, Chang et al. (2009) and Ren et al. (2012) showed significantly lower T-cell responses in HBV/HIV co- than HBV mono-infection [399, 458].

The present study investigates alterations in the HBV-specific T-cell immunity of HBV/HIV co-infected patients in comparison to individuals with chronic hepatitis B to determine mechanisms through which HIV can adversely affect HBV-related pathogenesis such as cell-mediated immunity, regulatory immune cells and mechanisms like T-cell exhaustion. The frequency and functionality of HBV antigen-reactive CD4 and CD8 T cells were therefore determined with flow cytometry-based intracellular cytokine staining. In addition to flow cytometric analysis of baseline T-cell immunity, the T-cell phenotype and T-cell dysfunction, cutting-edge bioinformatic analyses like tSNE (t-distributed stochastic neighbour embedding) and SPADE (spanning-tree progression analysis for density-normalized events) were performed to determine the phenotype of exhausted T cells in HBV/HIV co-infected patients.

Although HBV/HIV co-infected patients are not as plentiful, Sub-Saharan Africa is greatly threatened by the co-infection and efforts to further the understanding of the disease are vital. Since patients with HBV/HIV co-infection are burdened by two chronic diseases, relief of their symptoms and ongoing exploration of treatment and clinical care are essential. Broadening the knowledge regarding HBV/HIV co-infection might give new insight into the course of opportunistic infections in patients with HIV/AIDS, but especially into HBV-specific immunity, which determines the outcome of hepatitis B, and the immuno-pathological

mechanisms of hepatitis B. The findings might also spark the development of new antivirals for HBV and HIV or other HBV cure interventions, as well as improvements to currently investigated immunomodulatory therapy-approaches. These bear the opportunity to cure hepatitis B in the long run, if it is possible to mimic the mechanisms of resolution of acute hepatitis B or spontaneous clearance of the persistent infection, which is more prominent in the setting of HBV/HIV co-infection.

## 2. Results

For both, HBV as well as HIV infection, no curative treatment is available. While resolvers of acute HBV infection show a functional T-cell immunity, chronic hepatitis B is characterized by scarce, narrow-focused and dysfunctional T cells. HIV/AIDS is mainly described as an infection with progressive loss of CD4 T cells leading to immune suppression and increasing incidence of co-infections. In HBV/HIV co-infection, the aforementioned aspects of the immune system interact in a so-far not fully studied way. To enlighten the effects of HIV co-infection on the HBV-specific immunity, a biobank of PBMC samples of patients with HBV/HIV co-infection (n = 35) and HIV mono-infected subjects with simultaneous resolved hepatitis B (n = 8) was established.

PBMC of patients with t-HBV (treated HBV), HBV/HIV, HIV/rHBV (HIV infection and resolved HBV), HIV and tn-HBV (treatment-naïve HBV) infection, as well as of HC (healthy control; HBV/HIV naïve) subjects were used for the assays in this study. The cohort abbreviations, the infection and treatment status of the patients are presented in table 1.

Table 1: **Abbreviations of cohorts with infection and treatment specifications**

| Abbreviation | Cohort | HBV infection | HIV infection | Treatment |
|---|---|---|---|---|
| t-HBV | treated HBV mono-infected | persistent | never | NRTI |
| HBV/HIV | HBV & HIV co-infected | persistent | persistent | HBV-active ART |
| HIV/rHBV | HIV mono-infected & resolved HBV infected | resolved | persistent | HBV-active ART |
| HIV | HIV mono-infected | never | persistent | HBV-active ART |
| tn-HBV | treatment-naïve HBV mono-infected | persistent | never | never |
| HC | HBV/HIV naïve | never | never | never |

Analyzing differences in the socio-demographic and clinical parameters in the cohorts and determining the baseline immune status of the patients, a comparison of possible factors influencing T-cell immunity is presented. To determine if HIV co-infection has an impact on

HBV-specific T-cell immunity, the frequency and cytokine expression profile of HBV-reactive T cells was analyzed and compared to those of other virus-reactive T cells, T-cell reactivity was correlated with clinical data of the patients and finally immune regulatory cells and T-cell exhaustion was investigated.

## 2.1 Socio-demographic and clinical characteristics

Socio-demographic and clinical characteristics, which could cause differences in T-cell immunity, were compiled in table 2.

Characteristics of the cohorts were compared regarding socio-demographic variables revealing a similar percentage of sexes of patients with t-HBV, and HBV/HIV or HIV infection with 25 % females with 75 % males and 20 % females with 80 % males and 20 % females with 80 % males. By contrast, patients with HIV/rHBV or tn-HBV infection were different with 50 % and 57 % females and 50 % and 43 % males, respectively. The median age was similar with 47 (22 – 85, t-HBV), 49 (33 – 68, HBV/HIV), 45.5 (34 – 60, HIV/rHBV), 50 (24 – 74, HIV) and 45 (16 – 51, tn-HBV) years.

CD4 counts were only available for subjects with HBV/HIV, HIV/rHBV or HIV infection and were comparable with medians of 551 (105 – 1 579), 428 (60 – 1 302) and 620 (130 – 1 447) CD4 cells/µL. The CD4 : CD8 ratio of patients with t-HBV or tn-HBV infection was comparable with 1 : 0.6 and 1 : 0.7, respectively, while it was 1 : 1.7, 1 : 2.3 and 1 : 1.9 for patients with HBV/HIV, HIV/rHBV or HIV infection, respectively.

Table 2: **Socio-demographic and clinical characteristics** of patients with t-HBV, HBV/HIV, HIV/rHBV, HIV or tn-HBV infection

| | | t-HBV (n = 30) | HBV/HIV (n = 30) | HIV/rHBV (n = 8) | HIV (n = 10) | tn-HBV (n = 8) |
|---|---|---|---|---|---|---|
| Demographic variables | | | | | | |
| Sex | Fraction F : M | 25 % : 75 % | 20 % : 80 % | 50 % : 50 % | 20 % : 80 % | 57 % : 43 % |
| Age [years] | Median (Range) | 47 (22 – 85) | 49 (33 – 68) | 45.5 (34 – 60) | 50 (24 – 74) | 45 (16 – 51) |
| Clinical variables | | | | | | |
| Treated | Fraction | 100 % | 100 % | 100 % | 100 % | 0 % |
| CD4 count [/µL] | Median (Range) | unknown | 551 (105 – 1 579) | 428 (60 – 1 302) | 620 (130 – 1 447) | unknown |
| CD4 : CD8 | Average ratio | 1 : 0.6 | 1 : 1.7 | 1 : 2.3 | 1 : 1.9 | 1 : 0.7 |
| HIV RNA Load [copies/mL] | Median (Range) | n/a | 40 (0 – 2 570) | 40 (0 – 86 822) | 0 (0 – 209) | n/a |
| HBV DNA Load [IU/mL] | Median (Range) | 25 (0 – 942) | 10 (0 – 1 826) | 0 (0 – 10) | n/a | 45 (16 – 51) |
| HBsAg quant [IU/mL] | Median (Range) | 2 212 (11 – 18 062) | 1 208 (1 – 33 752) | 0 (0 – 0) | n/a | 581 (9 – 14 284) |
| HBeAg positive | Fraction | 13 % | 17 % | 0 % | n/a | 0 % |
| ALT [U/L] | Median (Range) | 38 (12 – 106) | 29 (11 – 96) | 23 (17 – 41) | 24.5 (15 – 29) | 32 (22 – 75) |
| AST [U/L] | Median (Range) | 33 (18 – 77) | 25 (15 – 82) | 21.5 (15 – 34) | 24.5 (22 – 51) | 33.5 (19 – 45) |

The HIV RNA load of patients with HBV/HIV or HIV/rHBV or HIV infection was different with 40 (0 – 2 570) or 40 (0 – 86 822) or 0 (0 – 209) copies/mL, respectively. The median HBV DNA load was similarly low for patients with t-HBV, HBV/HIV or tn-HBV infection at 25 (0 – 942) IU/mL, 10 (0 – 1 826) IU/mL and 45 (16 – 51) IU/ml. Respective median quantitative HBsAg was 2 212 (11 – 18 062) IU/mL, 1 208 (1 – 33 752) IU/mL and 581 (9 – 14 284) IU/mL for patients with t-HBV, HBV/HIV or tn-HBV infection, which shows a great difference in peripheral antigen levels. Quantitative HBsAg was below the detection limit (10 IU/mL) in patients with HIV/rHBV infection. Similar shares of patients with t-HBV (13 %) and HBV/HIV (17 %) infection were HBeAg positive, while patients with HIV/rHBV or tn-HBV infection were negative.

The serum levels of the liver enzyme ALT were comparable being 29 (11 – 96) U/L, 23 (17 – 41) U/L and 24.5 (15 – 29) U/L for patients with HBV/HIV, HIV/rHBV or HIV infection, respectively (Supplemental Figure 1). Serum ALT was significantly higher with 38 (12 – 106) U/L in patients with t-HBV than with HBV/HIV ($p = 0.0435$), HIV/rHBV ($p = 0.0251$) or HIV ($p = 0.0045$) infection. ALT levels were high in patients with tn-HBV infection at 38 (12 – 106) U/L. AST levels of patients with HBV/HIV, HIV/rHBV or HIV infection were similar at 25 (15 – 82) U/L, 21.5 (15 – 34) U/L and 24.5 (22 – 51) U/L, respectively. AST levels of patients with t-HBV infection at 33 (18 – 77) U/L were significantly higher than of patients with HBV/HIV ($p = 0.0243$), HIV/rHBV ($p = 0.0022$) and HIV ($p = 0.0424$) infection. Serum AST levels of patients with tn-HBV infection were significantly higher ($p = 0.0237$) than with HIV/rHBV infection with a median of 33.5 (19 – 45) U/L. Overall, serum levels of liver enzymes ALT and AST were similar in female, but significantly different in male patients (Supplemental Figure 2).

The patients' origin is a socio-demographic characteristic, which gives information about a possible bias in demographic, cultural or socioeconomic background and can imply the patients' genotype as the prevalence is different around the world [146]. The origin of the patients of the cohorts with t-HBV or HBV/HIV infection is compared in Figure 12.

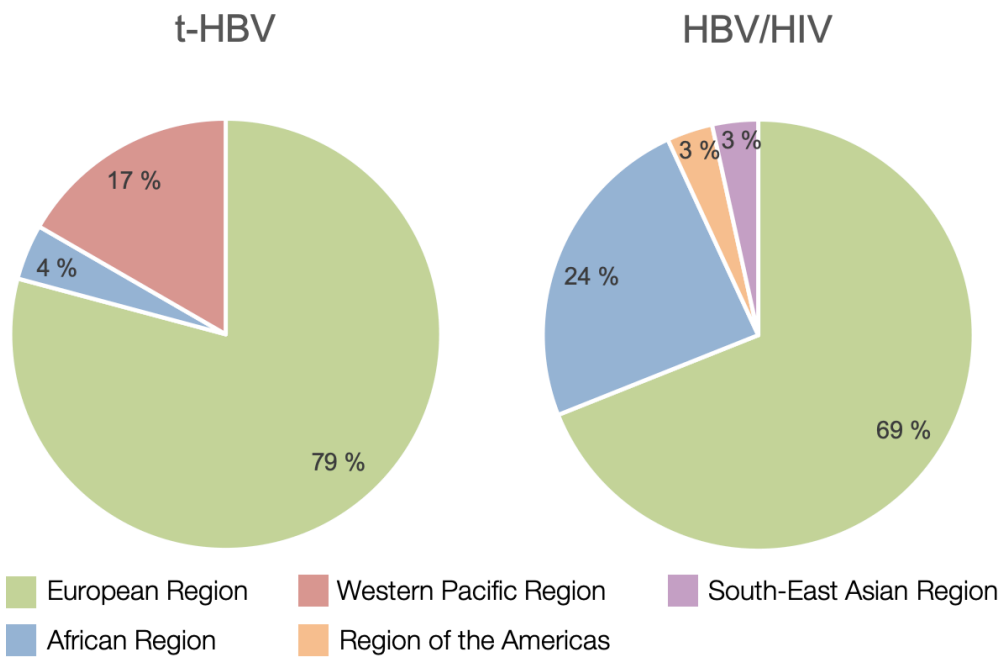


Figure 12: **Relative distribution of geographical origin** of patients with t-HBV or HBV/HIV infection according to WHO regions. Patients origin from the European region (tilia), the African region (cerulean), the Western Pacific region (cardinal), the region of the Americas (coral) and the South-East Asian region (lilac).

The patients' country of origin was compared between the cohorts of patients with t-HBV or HBV/HIV infection. The origin was distinguished according to WHO regions. While the fraction of patients from the European region was similar, patients with t-HBV infection stem more prominently from the Western Pacific (17 % to 0 %) and none originated in South-East Asia (0 % to 3 %). Only patients with HBV/HIV infection came from the Americas (3 % to 0 %). Interestingly, the origin of more subjects with HBV/HIV infection was Africa (24 % to only 4 %).

## 2.2 The baseline immune status is comparable in HBV mono- and HBV/HIV co-infection

The magnitude and cytokine pattern of HIV- and non-HIV specific immune responses can be affected by HIV infections' impact on the immune status of the patients regarding lymphocyte counts and frequencies [459], T-cell subsets [460] and activation status [461]. These parameters were therefore compared in patients with t-HBV, HBV/HIV, HIV/rHBV, HIV or tn-HBV infection and HBV/HIV naïve subjects.

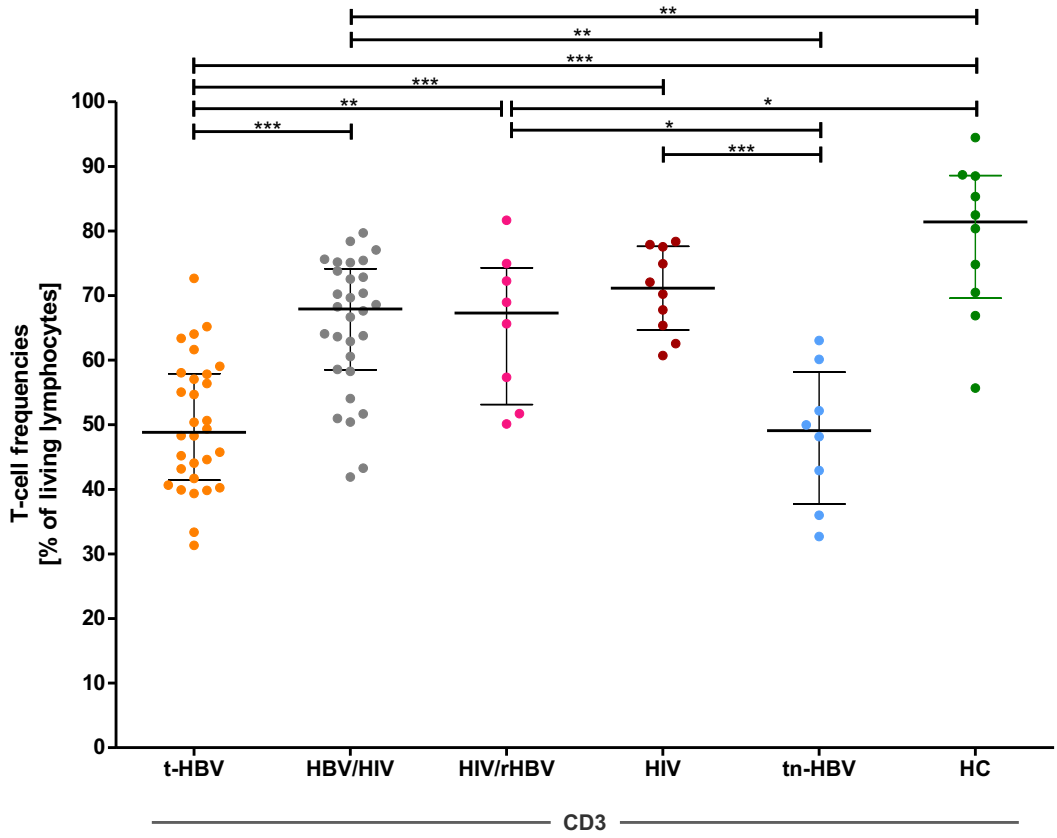


Figure 13: **CD3 T-cell frequency.** The frequency of CD3 T cells was detected by flow cytometry after staining of PBMC of patients with t-HBV (n = 30), HBV/HIV (n = 30), HIV/rHBV (n = 8), HIV (n = 10) and tn-HBV (n = 8) infection and of HBV/HIV naïve subjects (HC; n = 10). Median values and interquartile (25. and 75. percentile) range are indicated by the horizontal line and whiskers. (Primary Kruskall-Wallis test (ANOVA) with secondary Mann-Whitney U test, ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$)

Figure 13 displays CD3 T-cell frequencies determined by flow cytometry. Patients with t-HBV infection had significantly lower CD3 T-cell frequencies than subjects with HBV/HIV ($p < 0.0001$), HIV/rHBV ($p = 0.0031$) and HIV ($p < 0.0001$) infection and HBV/HIV naïve individuals ($p < 0.0001$). The median CD3 T-cell frequency was lower in patients with t-HBV (48.83 %, range 31.34 – 72.65 %, $p < 0.0001$), HBV/HIV (67.95 %, range 41.93 – 79.71 %, $p = 0.0039$), HIV/rHBV (67.31 %, range 50.14 – 81.68 %, $p = 0.0343$), HIV (71.16 %, range 60.73 – 78.38 %, non-significant) and tn-HBV (49.08 %, range 32.71 – 63.06 %, non-significant) infection than in HBV/HIV naïve subjects (81.41 %, range 55.67 – 94.47 %). Two groups of HBV- and/or HIV-infected patients were therefore identified showing on the one hand patients with t-HBV or tn-HBV infection having similar T-cell frequencies and on the other hand patients with HBV/HIV, HIV/rHBV or HIV infection to have similar T-cell frequencies.

Frequencies of peripheral CD4 and CD8 T cells were determined by flow cytometry (Figure 14) and CD4 T-cell frequencies showed significant correlations with CD4 counts/µL ($r^2_{HBV/HIV} = 0.6512$, $p_{HBV/HIV} < 0.0001$; $r^2_{HIV} = 0.6903$, $p_{HIV} = 0.0029$;), which were determined in whole blood of patients with HBV/HIV or HIV infection (Supplemental Figure 3).

The median CD4 T-cell frequencies were significantly higher ($p < 0.0001$) in patients with t-HBV (56.32 %, range 29.43 – 83.91 %) than with HBV/HIV (33.72 %, range 8.77 – 82.72 %) infection. CD4 T-cell frequencies of patients with t-HBV or tn-HBV (54.77 %, range 38.65 – 71.60 %) infection were similar to HBV/HIV naïve individuals (58.36 %, range 50.37 – 64.60 %). They were much lower for patients with HBV/HIV ($p = 0.0010$), HIV/rHBV (28.76 %, range 19.55 – 59.03 %, $p = 0.0021$) or HIV (32.82 %, range 15.57 – 68.82 %, $p = 0.0185$) infection.

Frequencies of CD8 T cells were significantly lower ($p < 0.0001$) in patients with t-HBV (36.30 %, range 12.76 – 62.63 %) than with HBV/HIV (57.31 %, range 16.31 – 83.27 %) infection. Patients with t-HBV or tn-HBV (38.44 %, range 24.59 – 53.39 %) infection had similarly low frequencies of CD8 T cells as HBV/HIV naïve subjects (33.42 %, range 26.08 – 44.78 %). By contrast CD8 T-cell frequencies of patients with HBV/HIV ($p = 0.0002$), HIV/rHBV (64.51 %, range 37.19 – 69.06 %, $p = 0.0003$) and HIV (63.91 %, range 25.17 – 74.36 %, $p = 0.0115$) infection were higher.

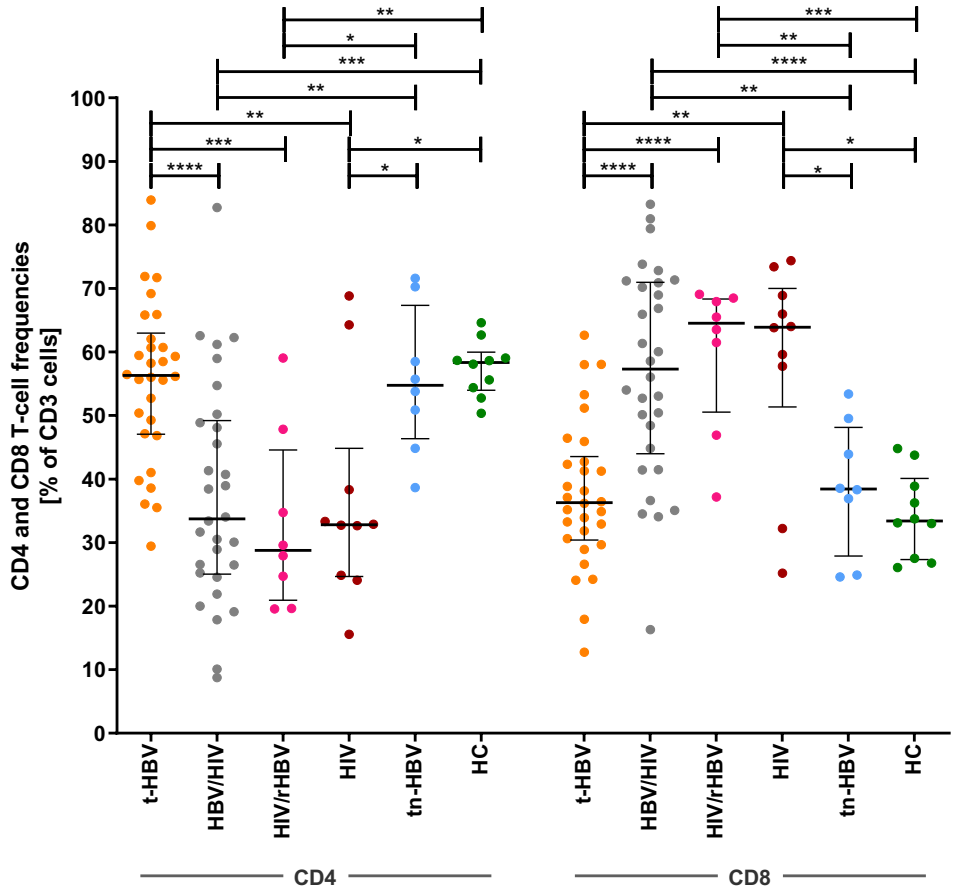


Figure 14: **CD4 and CD8 T-cell frequencies** were determined by flow cytometry. PBMC of patients with t-HBV (n = 30), HBV/HIV (n = 30), HIV/rHBV (n = 8), HIV (n = 10) and tn-HBV (n = 8) infection and of HBV/HIV naïve subjects (n = 10; HC) were stained with CD4 and CD8 detecting antibodies. Median values and interquartile (25. and 75. percentile) range are indicated. (Primary Kruskall-Wallis test (ANOVA) with secondary Mann-Whitney U test, ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$)

Besides CD4 and CD8 T-cell frequencies and CD4 : CD8 T-cell ratio, also T-cell subsets can relate to the function and antiviral activity of T cells. CD8 T-cell subsets defined by the expression of CD45RA and CCR7 were determined by flow cytometry. Figure 15 shows the distribution of naïve T cells ($T_N$; CD45RA$^+$ CCR7$^+$), central memory T cells ($T_{CM}$; CD45RA$^-$ CCR7$^+$), effector memory T cells ($T_{EM}$; CD45RA$^-$ CCR7$^-$) and terminal effector memory T cells ($T_{EMRA}$; CD45RA$^+$ CCR7$^-$) within the different cohorts.

Comparing frequencies of naïve CD8 T cells, patients with t-HBV (19.88 %, range 3.87 – 60.43 %) and HBV/HIV (14.76 %, range 4.92 – 42.15 %) infection had similar levels. Patients with t-HBV (non-significant) and HBV/HIV ($p$ = 0.0024) infection had lower frequencies of naïve CD8 T cells than HBV/HIV naïve individuals (48.23 %, range 8.46 – 69.04 %).

Central memory CD8 T cells were similarly frequent in patients with t-HBV (7.32 %, range 2.14 – 12.02 %) or HBV/HIV (7.36 %, range 1.23 – 22.63 %) infection and in comparison to HBV/HIV naïve subjects (5.75 %, range 3.02 – 12.20 %).

Frequencies of effector memory CD8 T cells were not significantly different between patients with t-HBV (34.37 %, range 19.09 – 69.39 %) and HBV/HIV (40.69 %, range 13.79 – 67.73 %) infection. Patients with t-HBV or HBV/HIV infection had higher, but not significantly different effector memory CD8 T-cell levels than HBV/HIV naïve subjects (25.82 %, range 13.52 – 46.89 %).

Similarly, frequencies of terminal effector memory CD8 T cells were higher in patients with HBV/HIV (34.18 %, range 12.07 – 78.09 %) than with t-HBV (22.03 %, range 11.69 – 71.70 %) infection, but were not significantly different. Terminal effector memory CD8 T-cell frequencies of HBV/HIV naïve subjects (18.45 %, range 10.99 – 48.26 %) were also lower.

Overall, frequencies of CD3, CD4 and CD8 T cells were significantly different between patients with t-HBV or HBV/HIV infection, although levels of T-cell subsets are comparable.

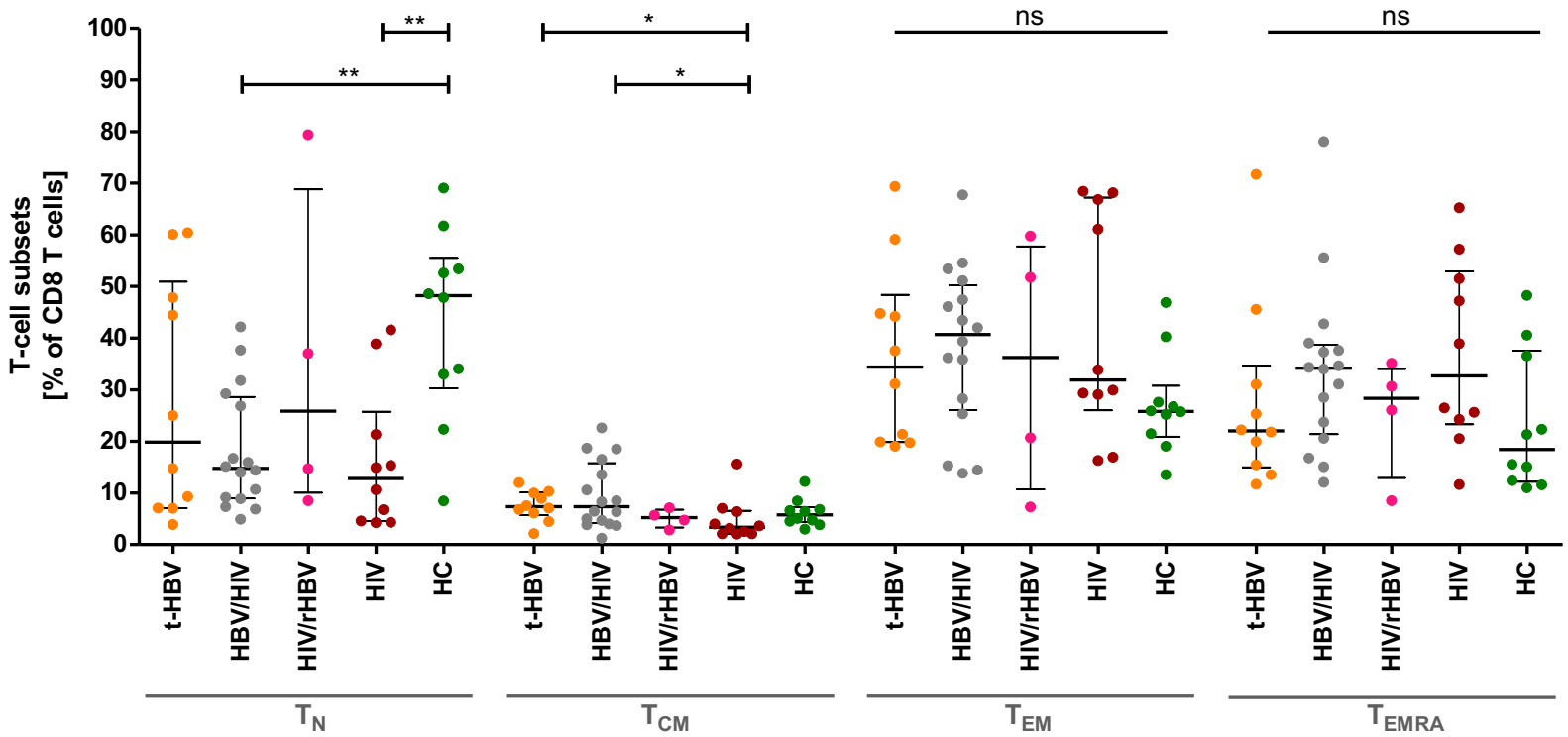


Figure 15: **CD8 T-cell subsets were analyzed by determination of the CD45RA and CCR7 expression** by flow cytometry of PBMC of patients with t-HBV (n = 10), HBV/HIV (n = 16), HIV/rHBV (n = 4) and HIV (n = 10) infection and HBV/HIV naïve subjects (HC; n = 10). Naïve T cells ($T_N$) were defined as $CD45RA^+$ $CCR7^+$, central memory T cells ($T_{CM}$) as $CD45RA^-$ $CCR7^+$, effector memory T cells ($T_{EM}$) as $CD45RA^-$ $CCR7^-$ and terminal effector memory T cells ($T_{EMRA}$) as $CD45RA^+$ $CCR7^-$. Median values and interquartile (25. and 75. percentile) range are indicated. (Primary Kruskall-Wallis test (ANOVA) with secondary Mann-Whitney U test, ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$)

Persistent immune activation is a hallmark of the pro-inflammatory environment in HIV infection and may affect the cytokine production of HBV-specific T cells in the coinfection. Levels of the markers PD-1 or CD25, which are upregulated upon activation, were quantified by flow cytometry for CD4 (Figure 16) and CD8 (Figure 17) T cells.

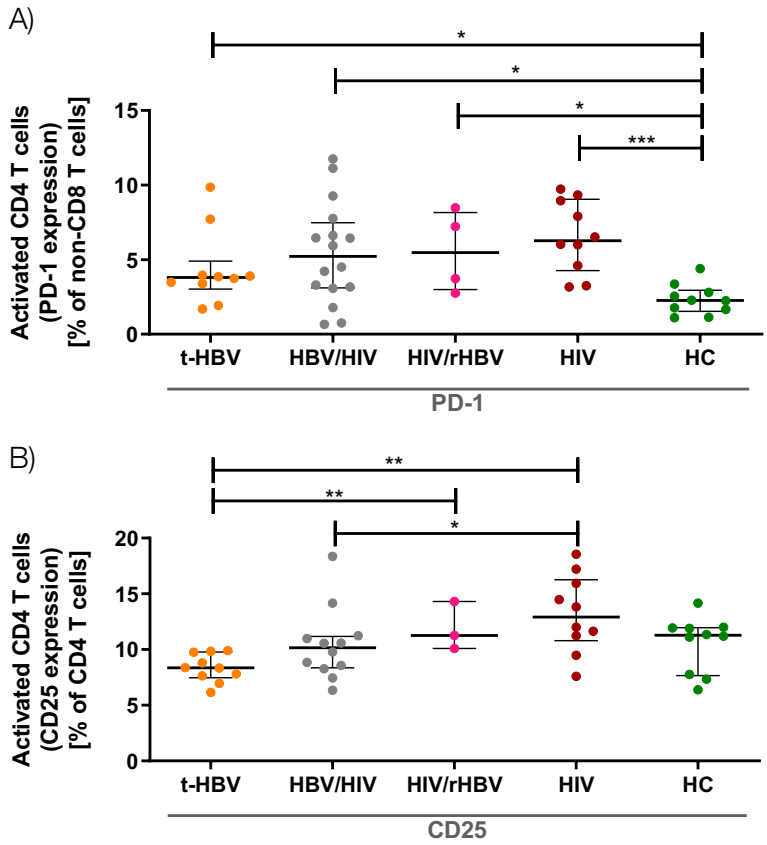


Figure 16: **Activated CD4 T cells. A) PD-1 and B) CD25 expression on CD4 T cells** of patients with t-HBV (n = 10), HBV/HIV (n = 16 and 12 respectively), HIV/rHBV (n = 4 and 3 respectively) and HIV (n = 10) infection and HBV/HIV naïve subjects (HC; n = 10). Median values and interquartile (25. and 75. percentile) range are indicated. (Primary Kruskall-Wallis test (ANOVA) with secondary Mann-Whitney U test, ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$)

Patients with t-HBV (3.80 %, range 1.69 – 9.86 %) or HBV/HIV (5.22 %, range 0.66 – 11.74 %) infection had similar frequencies of PD-1 producing CD4 T cells (Figure 16). Patients with t-HBV ($p = 0.0185$) or HBV/HIV ($p = 0.0143$) infection had significantly higher median frequencies of PD-1 producing CD4 T cells than HBV/HIV naïve individuals (2.27 %, range 1.11 – 4.40 %). Median $CD25^+$ CD4 T-cell frequencies were similar in patients with t-HBV (8.36 %, range 6.15 – 9.90 %) or HBV/HIV (10.17 %, range 6.35 – 18.36 %) infection and overall comparable to HBV/HIV naïve subjects (11.27 %, range 6.39 – 14.16 %).

Patients with t-HBV (3.57 %, range 2.11 – 5.03 %) or HBV/HIV (3.69 %, range 2.10 – 13.69 %) infection had similar levels of PD-1 producing CD8 T cells (Figure 17). PD-1 expression levels on CD8 T cells were significantly higher in patients with t-HBV ($p = 0.0039$) and HBV/HIV ($p = 0.0006$) infection in comparison to HBV/HIV naïve subjects (2.01 %, range 1.25 – 3.23 %).

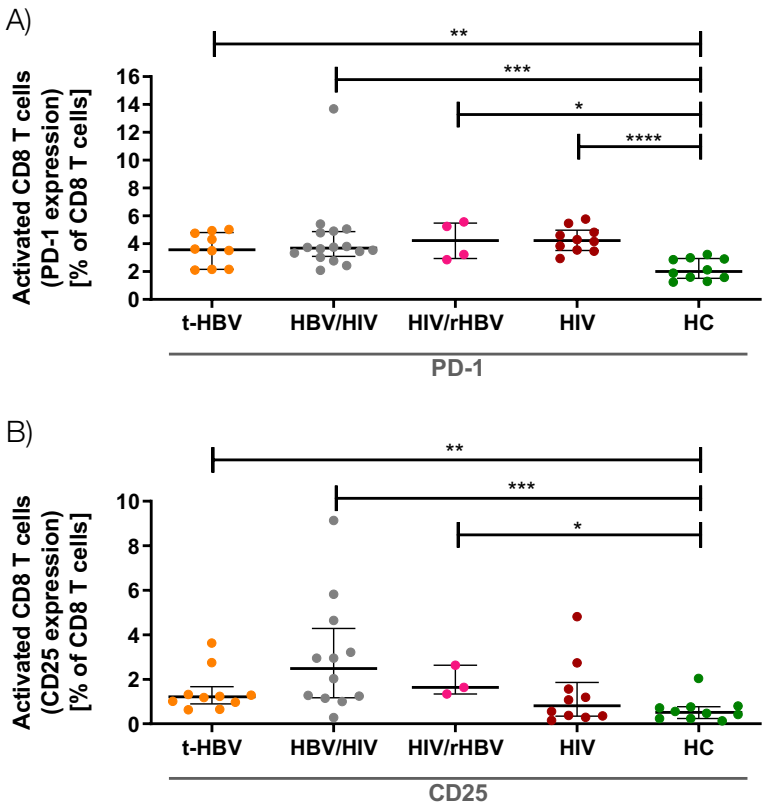


Figure 17: **Activated CD8 T cells. A) PD-1 and B) CD25 expression on CD8 T cells** of patients with t-HBV (n = 10), HBV/HIV (n = 16 and 12 respectively), HIV/rHBV (n = 4 and 3 respectively) and HIV (n = 10) infection and HBV/HIV naïve subjects (HC; n = 10). Median values and interquartile (25. and 75. percentile) range are indicated. (Primary Kruskall-Wallis test (ANOVA) with secondary Mann-Whitney U test, ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$)

Median $CD25^+$ CD8 T-cell frequencies were higher in patients with HBV/HIV (2.49 %, range 0.28 – 9.14 %) than with t-HBV (1.21 %, range 0.63 – 3.63 %) infection. Levels were significantly higher in patients with t-HBV ($p = 0.0052$) or HBV/HIV ($p = 0.0017$) infection than in HBV/HIV naïve subjects (0.51 %, range 0.13 – 2.04 %).

## 2.3 The cytokine expression profile of HBV-reactive T cells is impaired in HIV co-infection

The hypothesis that HIV co-infection affects HBV-specific T-cell immunity was tested by analyzing the response rates, the T-cell frequencies and the cytokine expression patterns of HBV-specific T cells (after *in vitro* restimulation) comparing individuals with or without HIV co-infection. To determine the reactivity and function of the T cells, PBMC were stimulated with HBV core, env and pol-derived peptide pools and intracellular cytokine staining (ICS) was performed detecting IFN-γ, IL-2 and TNF.

A Flu-, EBV- and CMV-derived peptide pool (FEC) that is optimized to challenge CD8 T-cell responses and an HIV nef-derived peptide pool were used as comparative antigens.

Figure 18 shows that HBV core- and env-specific CD4 T-cell response rates were higher in patients with HBV/HIV than t-HBV infection (77 % vs. 60 % and 70 % vs. 50 %, respectively), while pol-specific CD4 T-cell response rates were lower (50 % vs. 63 %). In comparison, FEC-specific CD4 T-cell response rates were higher in patients with HBV/HIV than with t-HBV (75 % vs. 64 %) infection. A particular focus should be on the fact that HBV env-specific CD4 T-cell response rates of patients with t-HBV infection were about 10 % less in contrast to challenge with HBV core-, pol-derived or FEC peptide pool. Interestingly, HBV pol-specific CD4 T-cell response rates of patients with HBV/HIV infection were 20 % less than upon HBV core, env and FEC stimulation.

Compared to patients with t-HBV infection, lower CD8 response rates for HBV core- (60 % vs. 67 %) and HBV pol-derived peptide pools (57 % vs. 70 %), but higher for HBV env-derived peptide pools (60 % vs. 50 %) were found within the cohort of patients with HBV/HIV infection. Fascinatingly, HBV env-specific CD8 T-cell response rates of patients with t-HBV infection were 20 % lower than for HBV core- or pol-specific CD8 T cells. FEC-specific CD8 T-cell response rates of patients with HBV/HIV or t-HBV infection were similar to each other (90 % and 96 % responders, respectively), but a lot higher than upon HBV-derived stimulations.

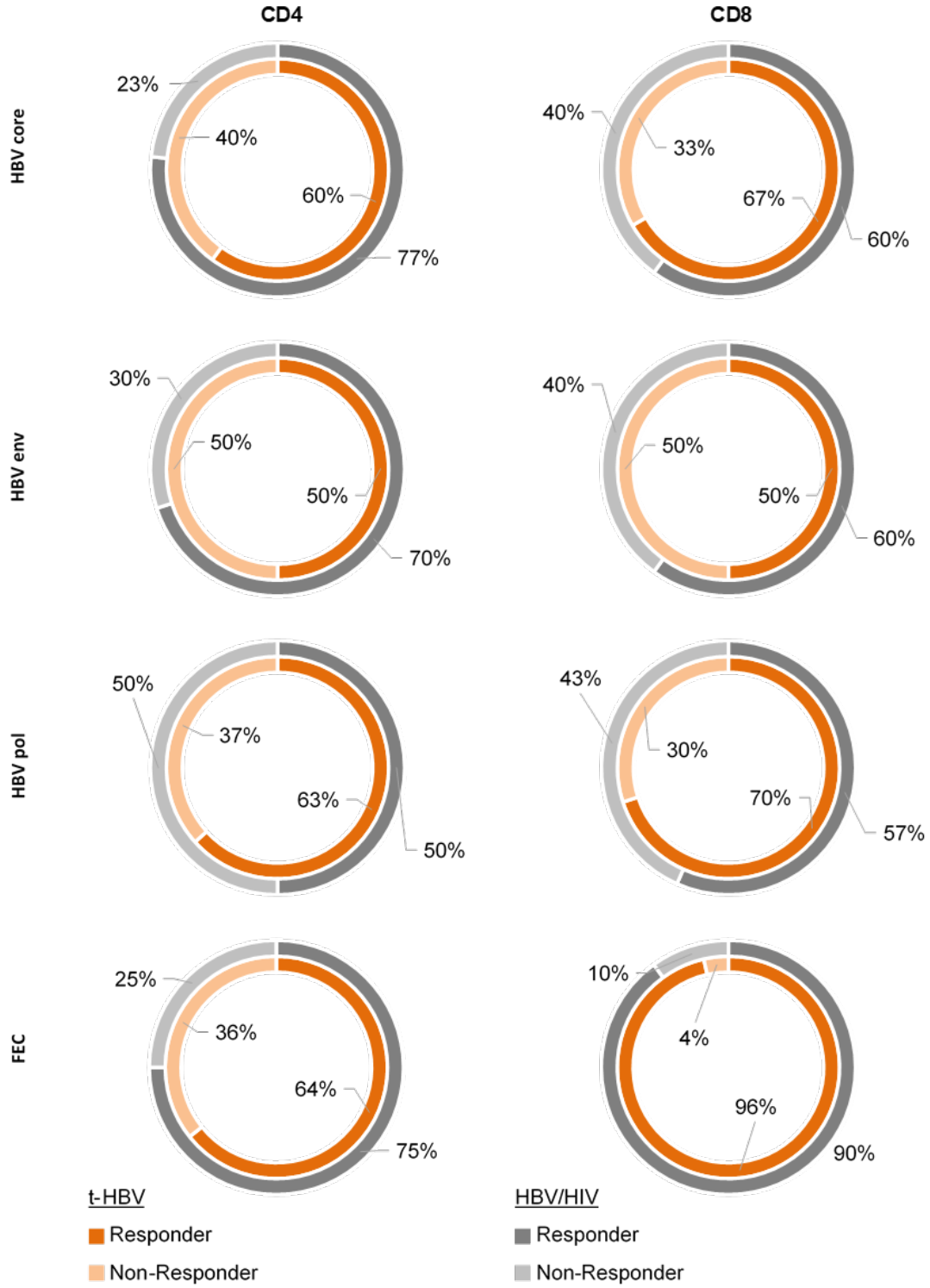


Figure 18: **Antigen-specific T-cell response rates.** Response rates of CD4 and CD8 T cells upon stimulation are represented as doughnut charts for patients with t-HBV (n = 30) or HBV/HIV (n = 30) infection. PBMC were stimulated with HBV protein-derived peptide pools as well as a peptide pool of the comparative antigen FEC and ICS (IFN-γ, IL-2, TNF) was performed. Patients with responses higher than the unstimulated control were determined as responders for the respective peptide pool.

Detection of T-cell responses showed that the stimulation and the intracellular cytokine staining were successful. Nevertheless, the response rates reveal that not all patients react to every stimulation, while T cells of every patient produced any cytokine in any stimulation (not shown). Overall, CD4 T-cell response rates of patients with HBV/HIV infection were higher, whereas response rates of CD8 T cells were lower, than of patients with t-HBV infection. Differences were not found in the control antigen FEC implying no underlying impairment of T-cell immunity in these patients.

### 2.3.1 The cytokine expression of HBV-reactive CD4 T cells

In addition to the overall T-cell response rate also differences in the magnitude (i.e. frequency) of HBV-specific T cells within the cohorts of patients with t-HBV or HBV/HIV infection were determined.

In the following, the frequency of HBV antigen-reactive CD4 T cells upon stimulation with different HBV protein-derived peptide pools (core, envelope and polymerase) was analyzed. Frequencies of HBV-reactive, cytokine (IFN-γ, IL-2, TNF) secreting CD4 T cells were determined by flow cytometry-based intracellular cytokine staining (Figure 19).

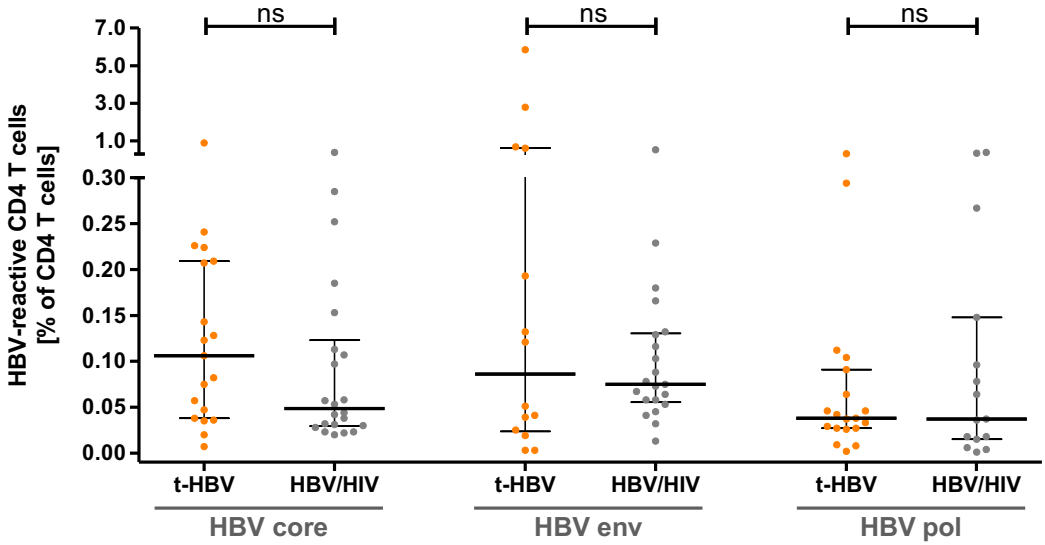


Figure 19: **HBV antigen-reactive CD4 T cells.** HBV core-, env- and pol-derived peptide pool stimulations challenged cytokine production by CD4 T cells, which were determined by flow cytometry-based intracellular cytokine staining (IFN-γ, IL-2 and TNF) of PBMC of patients with t-HBV (n = 30) and HBV/HIV (n = 30) infection. Median values and interquartile (25. and 75. percentile) range are indicated. (Mann-Whitney U test, ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$)

The median frequency of HBV core-reactive CD4 T cells was similar in patients with t-HBV (0.04 %, range 0.00 – 0.90 %) or HBV/HIV (0.03 %, range 0.00 – 0.40 %) infection. The cohort of patients with t-HBV infection (0.00 %, range 0.00 – 5.85 %) showed lower median HBV env-reactive CD4 T-cell frequencies than patients with HBV/HIV infection (0.06 %, range 0.00 – 0.53 %). HBV pol-reactive CD4 T cells were more frequent in patients with t-

HBV (0.03 %, range 0.00 – 0.32 %) than with HBV/HIV (0.001 %, range 0.00 – 0.39 %) infection. Most frequencies of CD4 T-cell responses were low, while high responders (frequencies higher than 1 %) were only found upon HBV env stimulation of PBMC of patients with t-HBV infection (2 out of 30 patients). The antigen hierarchy of CD4 T cells was dominated by HBV core over env over pol for patients with t-HBV infection, but defined by HBV env over core and pol for subjects with HBV/HIV infection.

Overall, CD4 T-cell frequencies were similar regarding HBV core-, env- and pol-reactive cells between t-HBV and HBV/HIV patients and no significant difference was found.

The data showed so far that patients with t-HBV or HBV/HIV infection have no significant differences in frequencies of total HBV-reactive CD4 T cells. To go into more detail, also differences in the distribution of HBV-reactive CD4 T cells producing IFN-γ, IL-2 and TNF were compared for patients with t-HBV or HBV/HIV infection (Figure 20).

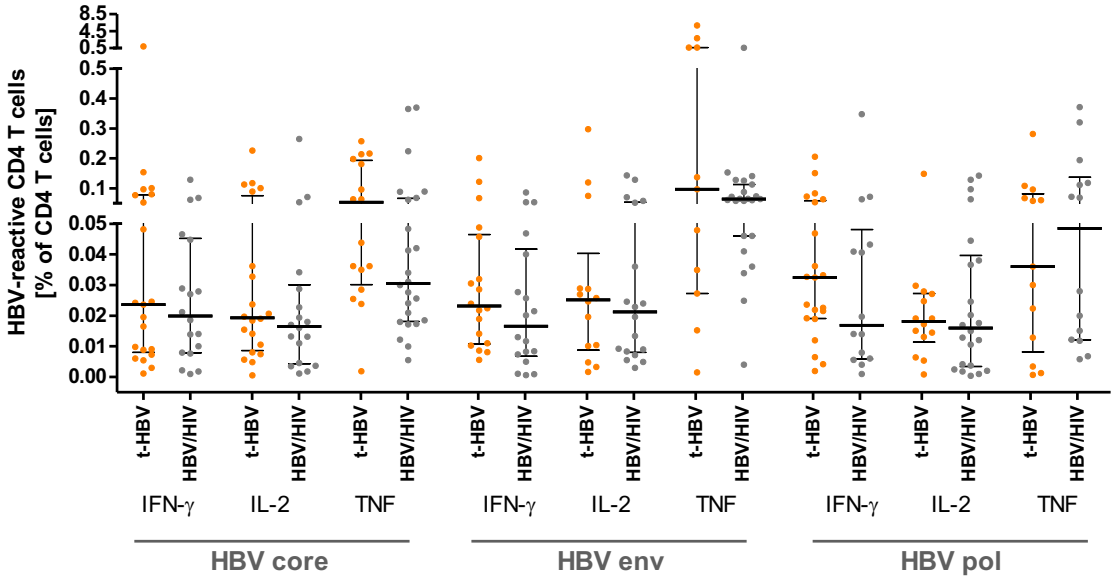


Figure 20: **Cytokine production of HBV antigen-reactive CD4 T cells.** Frequency of cytokine-producing CD4 T cells was determined by flow cytometry-based intracellular cytokine staining (IFN-γ, IL-2 and TNF) of PBMC of patients with t-HBV (n = 30) and HBV/HIV (n = 30) infection upon stimulation with HBV core-, env- and pol-derived peptide pools. Frequencies of any cytokine-producing population were not significantly different. Only values > 0 are shown. Median values and interquartile (25. and 75. percentile) range are indicated. (Mann-Whitney U test, ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$)

HBV core-, env- and pol-reactive CD4 T-cell frequencies of cells producing IFN-γ and IL-2 were comparable in patients with t-HBV or HBV/HIV infection. Median frequencies of HBV core-reactive CD4 T cells producing TNF (0.05 %, range 0.002 – 0.26 % vs. 0.03 %, range 0.006 – 0.37 %) were higher for patients with t-HBV than with HBV/HIV infection, but not significant. HBV env-reactive CD4 T cells producing TNF (0.10 %, range 0.002 – 5.86 % vs. 0.06 %, range 0.004 – 0.60 %) had higher median frequencies than IFN-γ and IL-2, but were also similar between patients with t-HBV or HBV/HIV infection. HBV pol-reactive CD4 T cells producing TNF (0.04 %, range 0.001 – 0.28 % vs. 0.05 %, range 0.006 – 0.37 %) were higher, but not significantly different in patients with HBV/HIV than t-HBV infection.

TNF-producing CD4 T cells were more frequent than IFN-γ and IL-2 producing T cells for any HBV antigen-reactive CD4 T cells. The median frequency of any cytokine-producing CD4 T cells independent of their specificity was higher in patients with t-HBV than with HBV/HIV infection except for polymerase-reactive TNF-producing CD4 T cells, which were higher in patients with HBV/HIV infection.

Neither frequencies of IFN-γ-, IL-2- nor TNF-producing HBV core-, env- or pol-reactive CD4 T cells were significantly different between t-HBV mono- and HBV/HIV co-infection.

Overall, cytokine production of CD4 T cells upon stimulation with HBV core, env or pol peptide pools was comparable between patients with t-HBV or HBV/HIV infection. Generally, TNF was the dominating cytokine in HBV-reactive CD4 T cells.

The degree of functionality, defined as the expression of one to multiple cytokines in parallel, of HBV-reactive CD4 T cells can co-determine the quality of T-cell responses. It is presented in Figure 21.

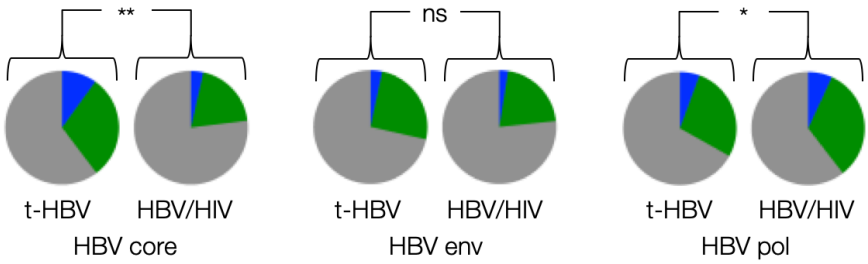


Figure 21: **Degree of functionality of HBV-reactive CD4 T cells.** Cytokine production was determined by flow cytometry-based intracellular cytokine staining upon stimulation with HBV core-, env- or pol-derived peptide pools for patients with t-HBV or HBV/HIV infection. Production of 1-3 cytokines in parallel is defined as mono- (grey), bi- (green) or trifunctional T cells (blue). (Permutation test, ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$)

Overall, the hierarchy of tri-, bi- and monofunctional HBV core-, env- or pol-reactive CD4 T cells was quite similar between the cohorts with monofunctional being the most dominant over bifunctional and eventually trifunctional CD4 T cells. The degree of functionality of HBV core-reactive ($p = 0.0017$) and pol-reactive ($p = 0.0074$) CD4 T cells was significantly different. No significant difference was found regarding the polyfunctionality of HBV env-reactive CD4 T cells ($p = 0.0878$).

Patients with t-HBV infection revealed more tri- and bifunctional HBV core-reactive CD4 T cells than patients with HBV/HIV infection. More HBV env-reactive bifunctional CD4 T cells were found in patients with t-HBV than with HBV/HIV infection. Less bifunctional HBV pol-reactive CD4 T cells were observed in patients with t-HBV than with HBV/HIV infection.

The bottom line is that the degree of functionality of HBV core- and HBV pol-reactive CD4 T cells is significantly different between patients with t-HBV or HBV/HIV infection.

While the overall cytokine production of HBV-reactive CD4 T cells was comparable between patients with t-HBV or HBV/HIV infection, the degree of functionality was significantly different for HBV core- and pol-specific cells. The cytokine expression profile of HBV antigen-reactive CD4 T cells was analyzed (Figure 22).

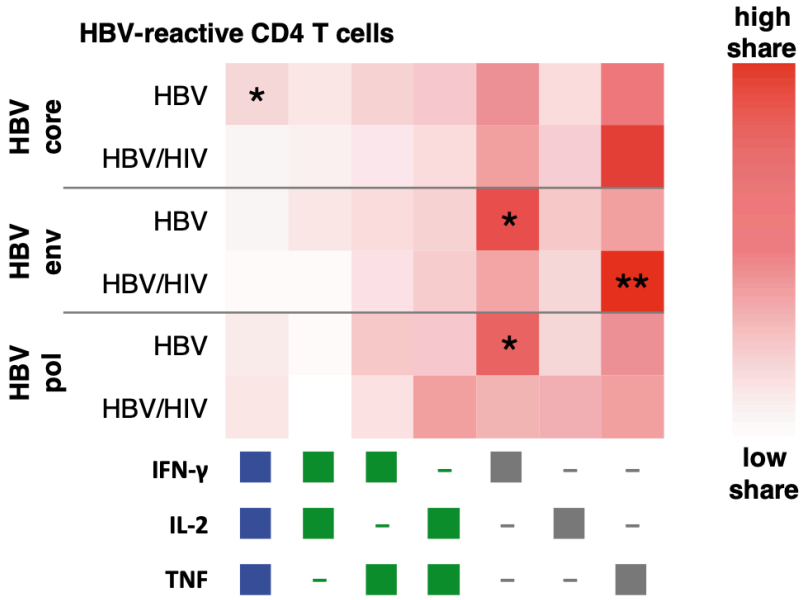


Figure 22: **Cytokine expression profile of HBV-reactive CD4 T cells.** Cytokine production (IFN-γ, IL-2, TNF) was determined by flow cytometry-based intracellular cytokine staining upon stimulation of PBMC with HBV core-, env- or pol-derived peptide pools from patients with t-HBV or HBV/HIV infection. The cytokine expression pattern is represented with dark red showing a high share of the antigen-reactive T-cell subpopulation of the respective patient cohort. Production of 1-3 cytokines in parallel is defined as mono- (grey), bi- (green) or trifunctional T cells (blue), which is indicated below the heatmap. Statistical significance was determined by comparing T-cell subsets producing the same cytokine combination in patients with t-HBV or HBV/HIV infection. (Student's t-test, ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$)

The cytokine expression profile shows the combination of cytokines IFN-γ, IL-2 and/or TNF produced by CD4 T cells in patients with t-HBV or HBV/HIV infection. Patients with t-HBV infection had a significantly higher share ($p = 0.0277$) of trifunctional HBV core-reactive CD4 T cells than patients with HBV/HIV infection. Although tri- and bifunctional HBV env- and pol-reactive CD4 T cells were not significantly different in patients with t-HBV or HBV/HIV infection, patients with t-HBV infection showed a significantly higher proportion of IFN-γ monofunctional HBV env-reactive cells ($p = 0.0481$) and significantly lower TNF monofunctional env-reactive T cells ($p = 0.0074$) than patients with HBV/HIV infection. IFN-γ

monofunctional HBV pol-reactive CD4 T cells were significantly more prevalent ($p = 0.0373$) in patients with t-HBV than with HBV/HIV infection.

In summary, the cytokine expression profile of HBV core-reactive CD4 T cells was more trifunctional in t-HBV than in HBV/HIV infection. By contrast, HBV pol-reactive CD4 T cells of patients with t-HBV infection were dominated by IFN-γ monofunctionality.

The cytokine expression profile, but not the frequency of HBV-reactive CD4 T cells was significantly different between t-HBV or HBV/HIV infection upon different HBV antigen-derived stimulations. As a concluding analysis, the cumulative HBV-reactive CD4 T cells were analyzed.

Frequencies of total HBV-reactive CD4 T cells were overall very low (Figure 23). Patients with t-HBV (0.10 %, range 0.00 – 6.14 %) or HBV/HIV (0.14 %, range 0.00 – 0.82 %) infection had similar ($p = 0.9764$) cumulative HBV-reactive CD4 T-cell frequencies.

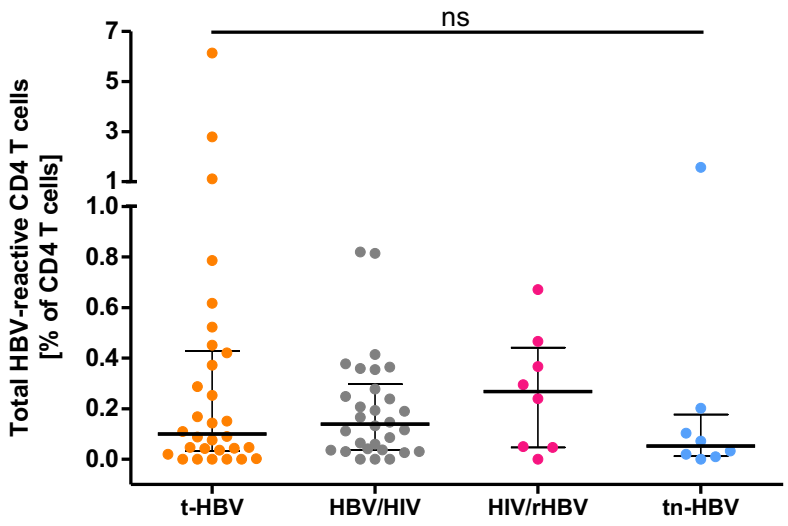


Figure 23: **Cumulative HBV-reactive CD4 T-cell responses.** HBV core-, env- and pol-derived peptide pool stimulations challenged cytokine production by CD4 T cells, which were determined by flow cytometry-based intracellular cytokine staining (IFN-γ, IL-2, TNF). Resulting frequencies were summed up to report overall HBV-reactive CD4 T cells of patients with t-HBV (n = 30), HBV/HIV (n = 30), HIV/rHBV (n = 8) or tn-HBV (n = 8) infection. Median values and interquartile (25. and 75. percentile) range are indicated. (Primary Kruskall-Wallis test (ANOVA) with secondary Mann-Whitney U test, ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$)

Analyzing reactive T cells to a secondary comparative antigen, which could be influenced by HBV co-infection, PBMC of patients with HBV/HIV or HIV infection were stimulated with an HIV nef-derived peptide pool and intracellular cytokine staining for the detection of IFN-γ, IL-2 and TNF was performed (Figure 24).

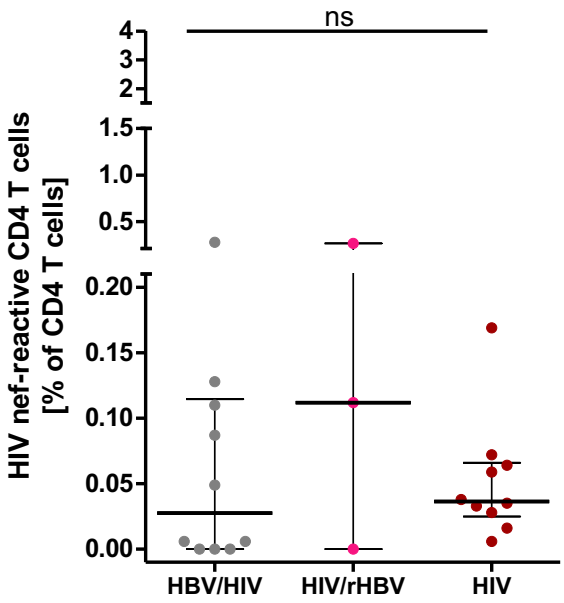


Figure 24: **HIV nef-reactive CD4 T cells.** Frequencies of cytokine-producing CD4 T cells of patients with HBV/HIV (n = 10), HIV/rHBV (n = 3) or HIV (n = 10) infection were detected. PBMC were stimulated with an HIV nef-derived peptide pool and flow cytometry-based ICS (IFN-γ, IL-2 and TNF) was performed. Median values and interquartile (25. and 75. percentile) range are indicated. (Mann-Whitney U test, ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$)

There was no significant difference of HIV nef-reactive CD4 T cells of patients with HBV/HIV or HIV infection with median frequencies of 0.03 % (range 0.002 – 0.33 %) and 0.04 % (range 0.01 – 0.17 %).

Patients with HBV/HIV infection had similar median HIV nef-reactive CD8 T-cell frequencies (0.09 %, range 0.02 – 2.21 %) as the cohort of patients with HIV infection (0.06 %, range 0.01 – 0.94 %).

Flow cytometry-based intracellular cytokine staining detected reactive CD4 T cells upon stimulation with the control antigen peptide pool FEC consisting of peptides from Flu, EBV and CMV (Figure 25).

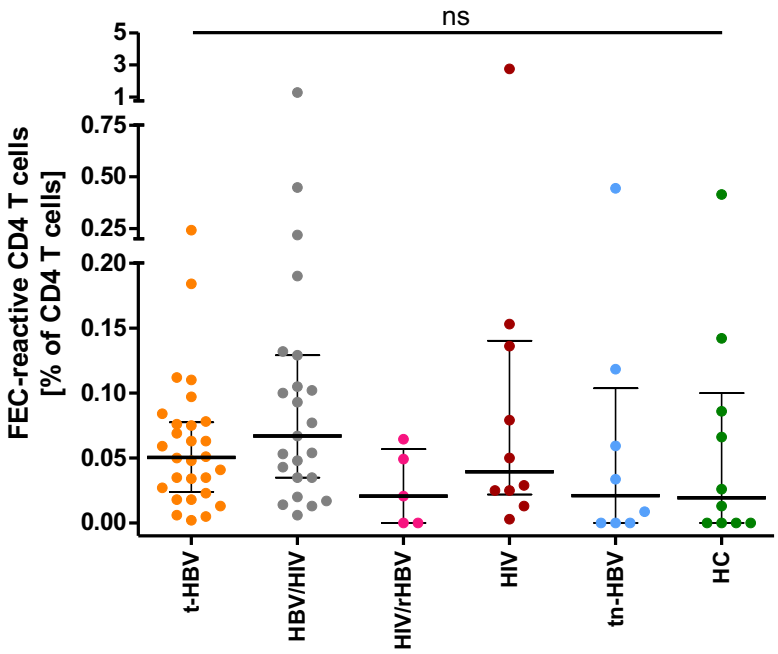


Figure 25: **FEC-reactive CD4 T cells were detected by flow cytometry.** PBMC of patients with t-HBV (n = 28), HBV/HIV (n = 23), HIV/rHBV (n = 5), HIV (n = 10) and tn-HBV (n = 8) infection and of HBV/HIV naïve subjects (n = 10, HC) were stimulated with a Flu-, EBV- and CMV-derived peptide pool as comparative antigens in detection of reactive T cells. Median values and interquartile (25. and 75. percentile) range are indicated. (Primary Kruskall-Wallis test (ANOVA), ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$)

FEC-reactive CD4 T-cell frequencies were similar between patients with t-HBV (0.05 %, range 0.002 – 0.24 %) and HBV/HIV (0.07 %, range 0.01 – 1.28 %) infection. Frequencies of HBV/HIV naïve subjects (0.02 %, range 0.00 – 0.42 %) were lower, but not significantly different, than of patients with t-HBV or HBV/HIV infection.

Interestingly, a high (frequencies > 0.75 %) and a low (< 0.75 %) responder group were observed in some cohorts. The high responder group was only found in the cohorts with HBV/HIV or HIV infection and only represented by one patient each.

No significant differences of the reactive CD4 T-cell frequencies were detected upon either the comparative, or the control stimulations with the HIV nef-derived or the FEC peptide pools between any of the analyzed cohorts.

Overall, frequencies of HBV-reactive CD4 T cells were comparable between patients with t-HBV or HBV/HIV infection, whereas the cytokine expression profile was significantly different regarding HBV core and pol-reactive CD4 T cells. In comparison to HBV/HIV infection, HBV core-reactive CD4 T cells were more trifunctional in patients with t-HBV infection, whereas pol-reactive CD4 T cells were dominated by IFN-γ monofunctional cells.

### 2.3.2 The cytokine expression of HBV-reactive CD8 T cells

Frequencies of HBV antigen-reactive CD8 T cells in patients with t-HBV or HBV/HIV infection were determined by flow cytometry-based intracellular cytokine staining detecting IFN-γ, IL-2 and TNF (Figure 26).

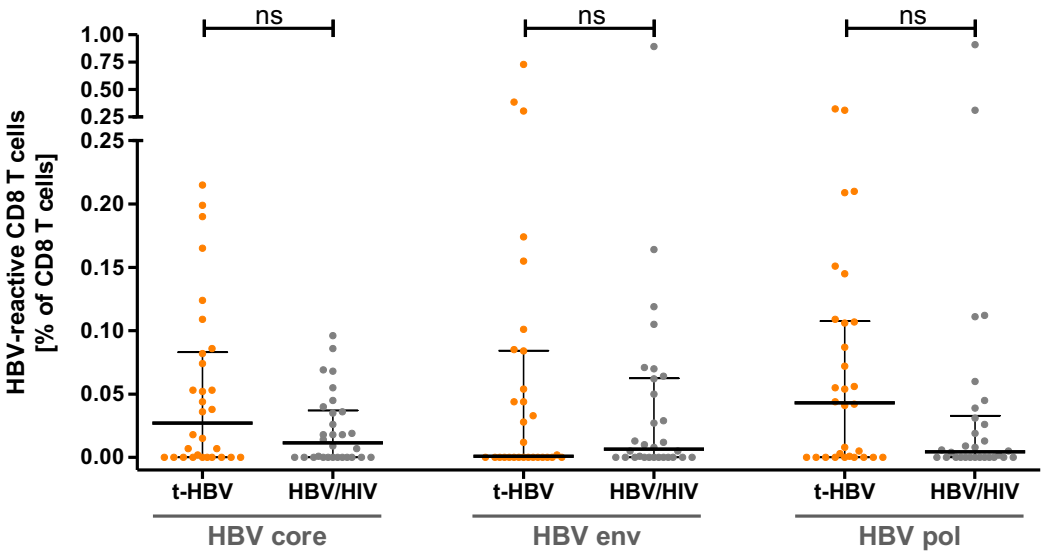


Figure 26: **HBV antigen-reactive CD8 T cells.** HBV core-, env- and pol-derived peptide pool stimulations challenged cytokine production by CD8 T cells, which were determined by flow cytometry-based intracellular cytokine staining (IFN-γ, IL-2 and TNF) of PBMC of patients with t-HBV (n = 30) and HBV/HIV (n = 30) infection. Median values and interquartile (25. and 75. percentile) range are indicated. (Mann-Whitney U test, ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$)

Patients with t-HBV infection had higher median HBV core-reactive CD8 T-cell frequencies (0.03 %, range 0.00 – 0.22 %) than subjects with HBV/HIV infection (0.01 %, range 0.00 – 0.10 %). HBV env-reactive CD8 T cells were less frequent in patients with t-HBV (0.001 %, range 0.00 – 0.73 %) than in HBV/HIV (0.01 %, range 0.00 – 0.89 %) infection. Frequencies of HBV pol-reactive CD8 T cells were higher in patients with t-HBV (0.04 %, range 0.00 – 0.32 %) than with HBV/HIV (0.005 %, range 0.00 – 0.91 %) infection. The frequency of HBV antigen-reactive CD8 T cells was not significantly different in cohorts of patients with t-HBV or HBV/HIV infection upon either stimulation with HBV core, env or pol derived peptide pools.

Although frequencies of HBV antigen-reactive CD8 T cells were not significantly different between patients with t-HBV or HBV/HIV infection, HBV antigen-reactive CD8 T cells that produce the cytokines IFN-γ, IL-2 or TNF were determined by flow cytometry-based intracellular cytokine staining (Figure 27).

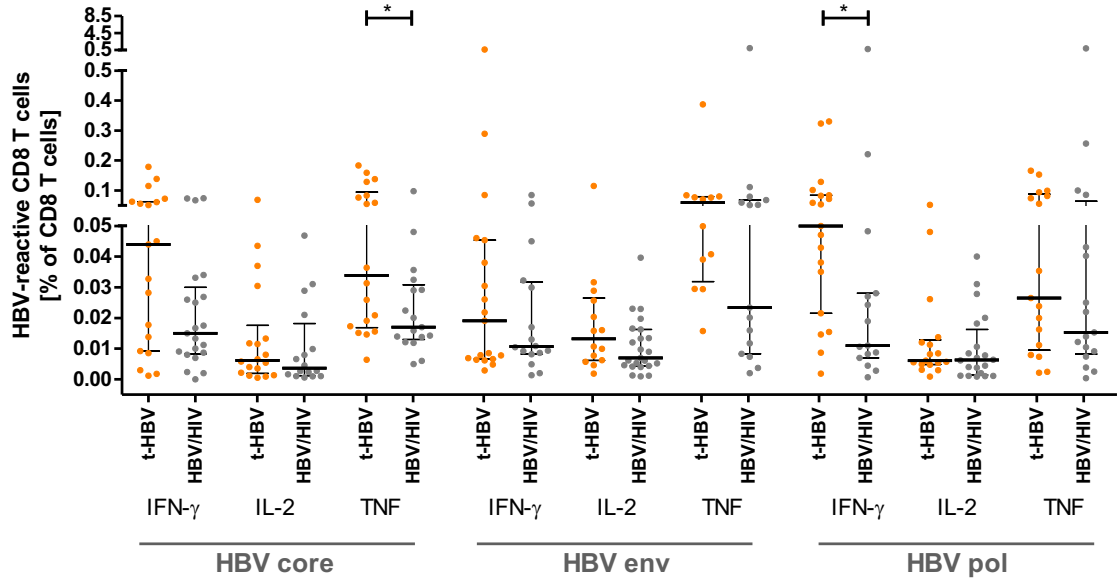


Figure 27: **Cytokine production of HBV antigen-reactive CD8 T cells.** Frequency of cytokine-producing CD8 T cells was determined by flow cytometry-based intracellular cytokine staining (IFN-γ, IL-2 and TNF) of PBMC of patients with t-HBV (n = 30) and HBV/HIV (n = 30) infection upon stimulation with HBV core-, env- and pol-derived peptide pools. Only values > 0 are shown. Median values and interquartile (25. and 75. percentile) range are indicated. (Mann-Whitney U test, ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$)

Median frequencies of HBV core-reactive CD8 T cells were higher in patients with t-HBV than with HBV/HIV infection regarding production of IFN-γ (0.04 %, range 0.001 – 0.18 % vs. 0.02 %, range 0.00003 – 0.07 %), IL-2 (0.006 %, range 0.0006 – 0.069 % vs. 0.004 %, range 0.0006 – 0.047 %) or TNF (0.034 %, range 0.006 – 0.18 % vs. 0.017 %, range 0.005 – 0.097 %).

Similarly, median HBV env-reactive CD8 T cells were also higher in patients with t-HBV than HBV/HIV infection regarding the production of IFN-γ (0.02 %, range 0.003 – 0.66 % vs. 0.01 %, range 0.001 – 0.09 %), IL-2 (0.01 %, range 0.002 – 0.12 % vs. 0.01 %, range 0.001 – 0.04 %) or TNF (0.06 %, range 0.02 – 0.39 % vs. 0.02 %, range 0.002 – 0.95 %), which was also true for HBV pol-reactive CD8 T cells producing IFN-γ (0.05 %, range 0.002

– 0.33 % vs. 0.01 %, range 0.001 – 0.73 %), IL-2 (0.01 %, range 0.001 – 0.05 % vs. 0.01 %, range 0.001 – 0.04 %) or TNF (0.03 %, range 0.002 – 0.17 % vs. 0.02 %, range 0.0004 – 0.90 %), which were more frequent in patients with t-HBV than with HBV/HIV infection.

IFN-γ-producing CD8 T cells were significantly more frequent in patients with t-HBV than with HBV/HIV infection upon stimulation with an HBV pol-derived peptide pool ($p = 0.0184$). IL-2 production was comparable between both cohorts in all stimulations. TNF-producing core-reactive CD8 T cells were significantly more frequent in patients with t-HBV than with HBV/HIV infection ($p = 0.0238$).

While frequencies of HBV antigen-reactive CD8 T cells were similar between patients with t-HBV or HBV/HIV infection, the frequency of TNF-producing HBV core- as well as IFN-γ-producing pol-reactive CD8 T cells were significantly higher in subjects with t-HBV than with HBV/HIV infection. This did not give any insight into the question if single cells did produce multiple cytokines in parallel, which is defined as the functionality of T cells. The degree of functionality of T cells is proposed to be a correlate of protection in infectious diseases [462] and was therefore evaluated (Figure 28).

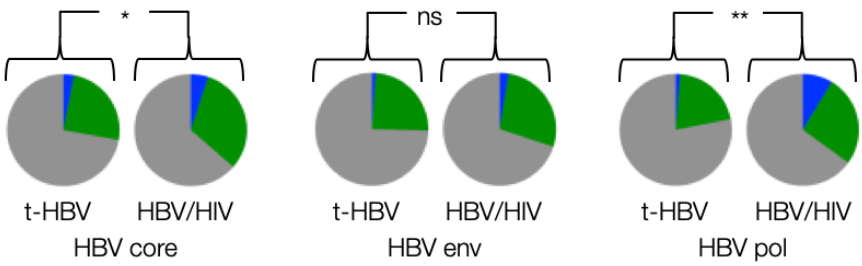


Figure 28: **Degree of functionality of HBV-reactive CD8 T cells.** Cytokine production was determined by flow cytometry-based intracellular cytokine staining upon stimulation with HBV core-, env- or pol-derived peptide pools for patients with t-HBV or HBV/HIV infection. Production of 1-3 cytokines in parallel is defined as mono- (grey), bi- (green) or trifunctional T cells (blue). (Permutation test, ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$)

The overall ratio of tri- to bi- to monofunctional T cells was quite similar in both cohorts upon all three HBV antigen-derived stimulations. Significantly more polyfunctional HBV core- and pol-reactive ($p$ = 0.0157) cells were found in patients with HBV/HIV infection. HBV env-reactive CD8 T cells did not reveal significantly different functionality ratios in patients with t-HBV or HBV/HIV infection.

While the degree of functionality was significantly different in HBV core- and HBV pol-reactive CD8 T cells between patients with t-HBV or HBV/HIV infection, the produced combination of cytokines was unclear. The cytokine expression pattern was determined showing the share of HBV-reactive CD8 T-cell populations that produce a combination of cytokines in patients with t-HBV or HBV/HIV infection (Figure 29).

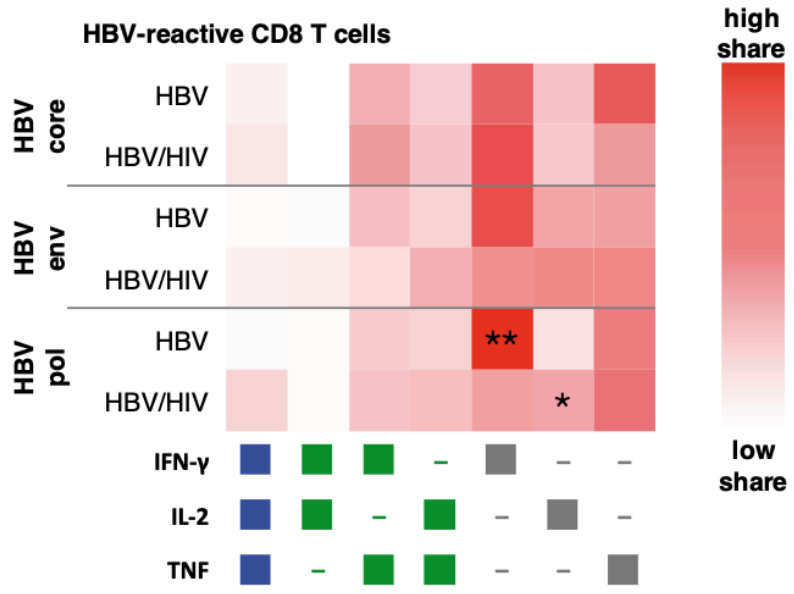


Figure 29: **Cytokine expression profile of HBV-reactive CD8 T cells.** Cytokine production (IFN-γ, IL-2, TNF) was determined by flow cytometry-based intracellular cytokine staining upon stimulation of PBMC with HBV core-, env- or pol-derived peptide pools from patients with t-HBV or HBV/HIV infection. The cytokine expression pattern is represented with dark red showing a high share of the antigen-reactive T-cell subpopulation of the respective patient cohort. Production of 1-3 cytokines in parallel is defined as mono- (grey), bi- (green) or trifunctional T cells (blue), which is indicated below the heatmap. Statistical significance was determined by comparing T-cell subsets producing the same cytokine combination in patients with t-HBV or HBV/HIV infection. (Student's t-test, ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$)

No difference in populations of tri- and bifunctional CD8 T cells of any reactivity and cohort were observed. HBV core- and env-reactive CD8 T cells had no significantly different shares of subpopulations between both cohorts. IFN-γ monofunctional HBV pol-reactive CD8 T cells were significantly more prevalent ($p = 0.0080$) in patients with t-HBV than HBV/HIV infection. By contrast, IL-2 monofunctional HBV pol-reactive CD8 T cells had a significantly higher share ($p = 0.0350$) in patients with HBV/HIV than with t-HBV infection.

So far, the data showed no significant differences in the frequency of HBV-reactive CD8 T cells between cohorts of patients with t-HBV or HBV/HIV infection, but significant alterations in the cytokine expression profile of HBV pol-reactive CD8 T cells. The frequency of total HBV-reactive CD8 T cells was determined by flow cytometry-based intracellular cytokine staining. Total HBV-reactive CD8 T-cell responses were calculated by summing up the frequency of CD8 T cells that produce IFN-γ, IL-2 and/or TNF upon any HBV protein-derived peptide pool stimulation (Figure 30).

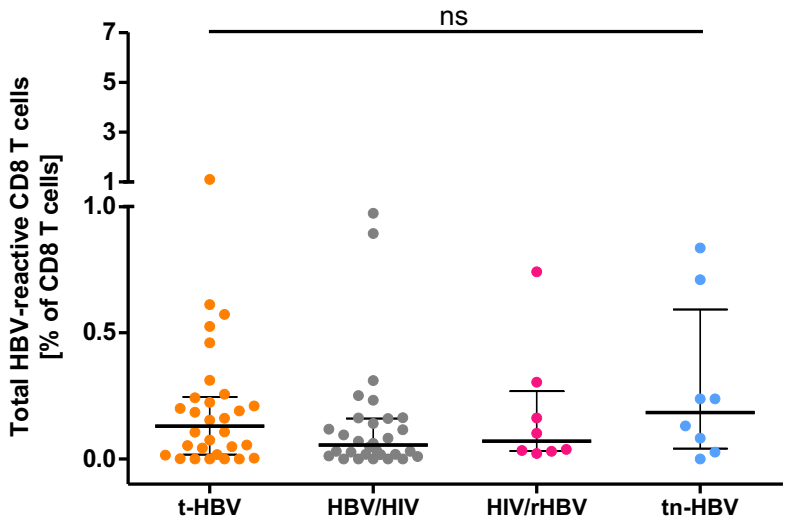


Figure 30: **Cumulative HBV-reactive CD8 T-cell responses.** HBV core-, env- and pol-derived peptide pool stimulations challenged cytokine production by CD8 T cells, which were determined by flow cytometry-based intracellular cytokine staining (IFN-γ, IL-2, TNF). Resulting frequencies were summed up to report overall HBV-reactive CD8 T cells of patients with t-HBV (n = 30), HBV/HIV (n = 30), HIV/rHBV (n = 8) or tn-HBV (n = 8) infection. Median values and interquartile (25. and 75. percentile) range are indicated. (Primary Kruskall-Wallis test (ANOVA) with secondary Mann-Whitney U test, ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$)

Median HBV-reactive CD8 T-cell frequencies were higher in patients with t-HBV (0.13 %, range 0.00 – 1.11 %) or HBV/HIV (0.06 %, range 0.00 – 0.97 %) infection. Subjects with HIV/rHBV (0.07 %, range 0.02 – 0.74 %) infection had similar levels as with HBV/HIV infection, whereas patients with tn-HBV (0.19 %, range 0.00 – 0.84 %) had the highest frequencies. Overall, there was no significant difference of HBV-reactive CD8 T cells between cohorts of patients with t-HBV or HBV/HIV infection.

Analyzing reactive T cells to a comparative antigen, which could be influenced by the co-infection, PBMC of patients with HBV/HIV or HIV infection were stimulated with an HIV nef-derived peptide pool and intracellular cytokine staining for the detection of IFN-γ, IL-2 and TNF was performed (Figure 31).

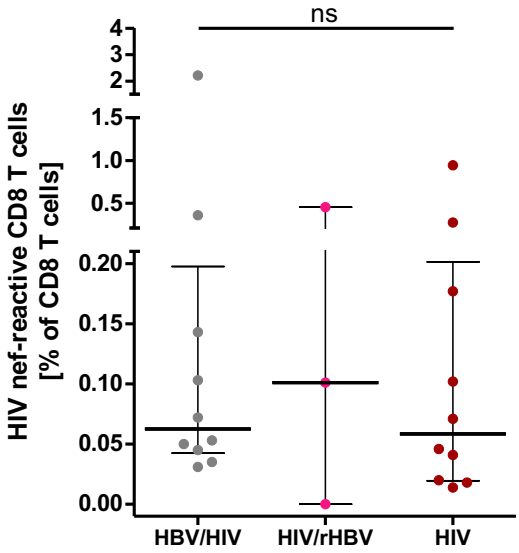


Figure 31: **HIV nef-reactive CD8 T cells.** Frequencies of cytokine-producing CD8 T cells of patients with HBV/HIV (n = 10), HIV/rHBV (n = 3) or HIV (n = 10) infection were detected. PBMC were stimulated with an HIV nef-derived peptide pool and flow cytometry-based ICS (IFN-γ, IL-2 and TNF) was performed. Median values and interquartile (25. and 75. percentile) range are indicated. (Mann-Whitney U test, ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$)

There was no significant difference of median HIV nef-reactive CD8 T-cell frequencies of patients with HBV/HIV (0.09 %, range 0.02 – 2.21 %) or HIV (0.06 %, range 0.01 – 0.94 %) infection.

Neither HBV antigen-reactive, nor HIV nef-reactive CD8 T-cell frequencies were significantly different in patients with t-HBV, HBV/HIV, HIV/rHBV, HIV or tn-HBV infection. As a control, CD8 T-cell responses upon stimulation with the comparative antigen peptide pool FEC were detected with flow cytometry-based ICS (Figure 32).

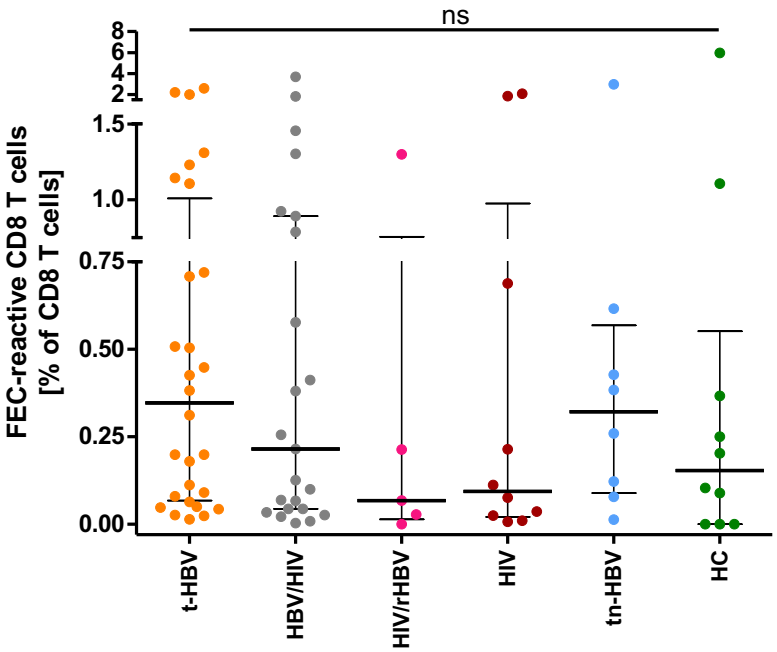


Figure 32: **FEC-reactive CD8 T cells were detected by flow cytometry.** PBMC of patients with t-HBV (n = 28), HBV/HIV (n = 23), HIV/rHBV (n = 5), HIV (n = 10) and tn-HBV (n = 8) infection and of HBV/HIV naïve subjects (n = 10, HC) were stimulated with a Flu-, EBV- and CMV-derived peptide pool as comparative antigens in detection of reactive T cells. Median values and interquartile (25. and 75. percentile) range are indicated. (Primary Kruskall-Wallis test (ANOVA), ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$)

Interestingly, a high (> 0.75 %) and low (< 0.75 %) responder group was observed in all cohorts. 25 % of patients with t-HBV and 30 % with HBV/HIV infection were high responders to FEC stimulation, which was only slightly lower in HBV/HIV naïve subjects with 20 %.

Median FEC-reactive CD8 T-cell frequencies were higher in patients with t-HBV (0.35 %, range 0.01 – 2.58 %) than with HBV/HIV (0.22 %, range 0.003 – 3.68 %) infection. The difference was not significant. FEC-reactive CD8 T-cell frequencies of HBV/HIV naïve individuals (0.15 %, range 0.00 – 5.97 %) were similar to those of patients with HBV/HIV infection and lower, but not significantly than of patients with t-HBV infection.

While frequencies of total HBV-reactive and HBV antigen-reactive CD8 T cells were comparable between patients with t-HBV or HBV/HIV infection, significant changes in the cytokine production and the cytokine expression profile were found. HBV core-reactive CD8 T cells of patients with t-HBV produced more TNF, while pol-reactive T cells produced more IFN-γ than of patients with HBV/HIV infection. Both HBV core- and pol-reactive CD8 T cells had a higher degree of polyfunctionality in patients with t-HBV than HBV/HIV infection.

### 2.3.3 The cytokine expression of all HBV-reactive (CD3) T cells

Neither HBV-reactive CD4, nor CD8 T-cell frequencies were significantly different between patients with t-HBV or HBV/HIV infection. Overall HBV-reactive CD3 T cells were determined with flow-cytometry based ICS (Figure 33).

Frequencies of total HBV-reactive CD3 T lymphocytes were similar in patients with t-HBV (0.14 %, range 0.005 – 4.38 %) or HBV/HIV (0.10 %, range 0.00 – 0.82 %) infection.

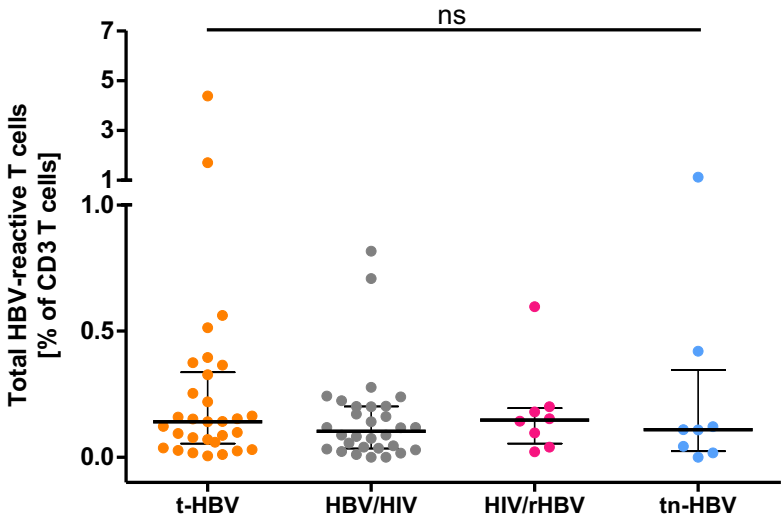


Figure 33: **Cumulative HBV-reactive CD3 T-cell responses.** HBV core-, env- and pol-derived peptide pool stimulations challenged cytokine production by CD4 and CD8 T cells, which were determined by flow cytometry-based intracellular cytokine staining (IFN-γ, IL-2, TNF). Resulting frequencies were summed up to report overall HBV-reactive CD3 T cells of patients with t-HBV (n = 30), HBV/HIV (n = 30), HIV/rHBV (n = 8) or tn-HBV (n = 8) infection. Median values and interquartile (25. and 75. percentile) range are indicated. (Primary Kruskall-Wallis test (ANOVA) with secondary Mann-Whitney U test, ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$)

While total HBV-reactive CD3 T-cell frequencies were not significantly different between patients with t-HBV or HBV/HIV infection, HBV antigen-reactive CD3 T cells were determined (Figure 34).

Frequencies of HBV core-, env- and pol-reactive CD3 T cells were comparable between patients with t-HBV or HBV/HIV infection. Patients with t-HBV infection had higher median frequencies of HBV core-reactive (0.05 %, range 0.002 – 0.36 % vs. 0.03 %, range 0.005 – 0.24 %) and pol-reactive (0.04 %, range 0.00 – 0.26 % vs. 0.02 %, range 0.001 – 0.65 %), but lower env-reactive (0.03 %, range 0.00 – 4.14 % vs. 0.04 %, range 0.003 – 0.58 %) CD3 T cells than subjects with HBV/HIV infection.

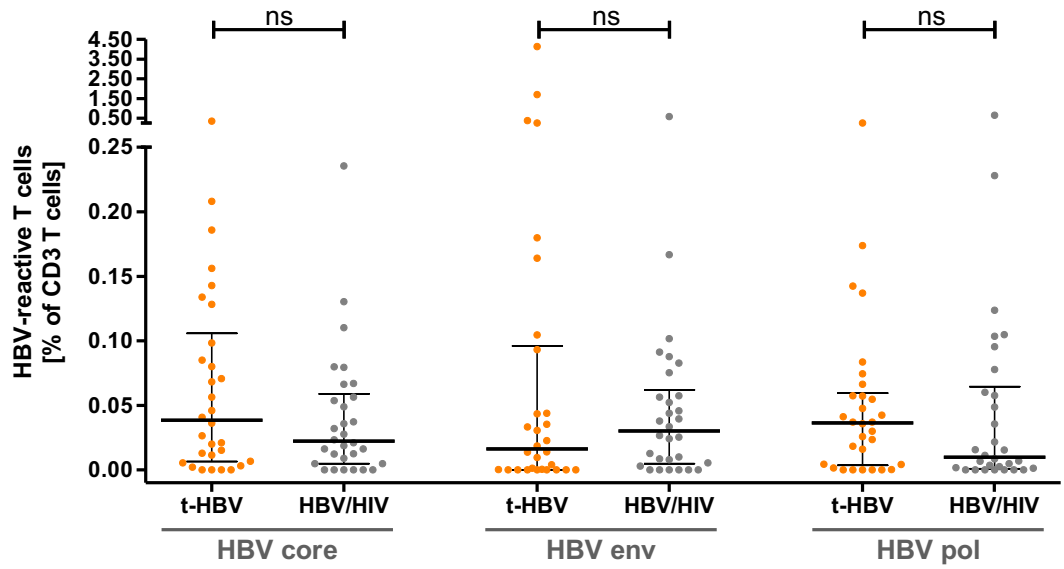


Figure 34: **HBV antigen-reactive CD3 T cells.** HBV core-, env- and pol-derived peptide pool stimulations challenged cytokine production by CD3 T cells, which were determined by flow cytometry-based intracellular cytokine staining (IFN-γ, IL-2 and TNF) of PBMC of patients with t-HBV (n = 30) and HBV/HIV (n = 30) infection. Median values and interquartile (25. and 75. percentile) range are indicated. (Mann-Whitney U test, ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$)

Total HBV-reactive and HBV antigen-reactive CD3 T-cell frequencies were comparable between patients with t-HBV or HBV/HIV infection. While this was also true for HBV antigen-reactive CD8 T cells, the cytokine production was significantly different. The cytokine production of HBV-reactive CD3 T cells was therefore analyzed (Figure 35).

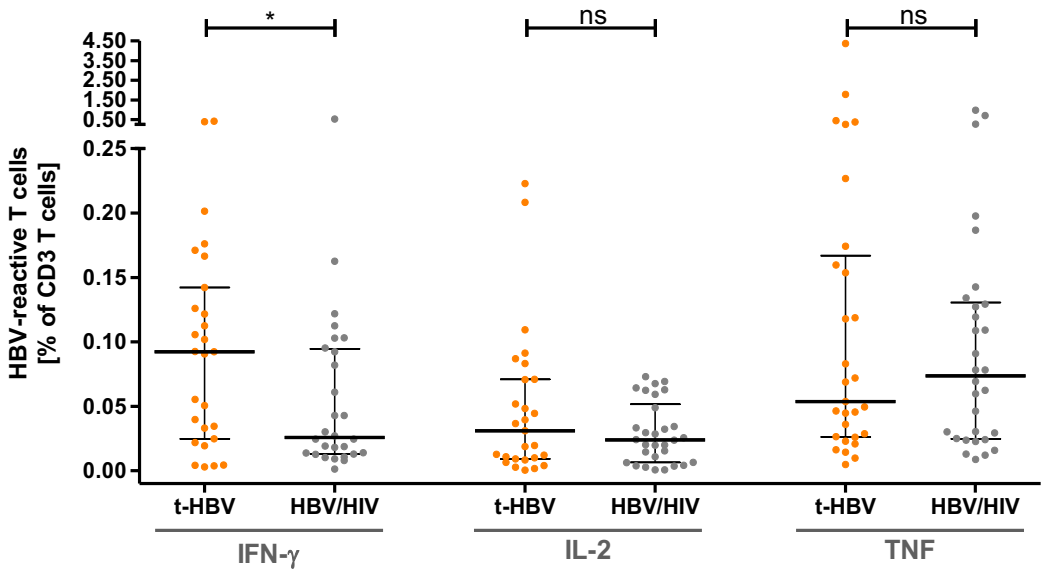


Figure 35: **Cytokine production of HBV-reactive CD3 T cells.** Frequency of cytokine-producing CD3 T cells was determined by flow cytometry-based intracellular cytokine staining (IFN-γ, IL-2 and TNF) of PBMC of patients with t-HBV (n = 30) and HBV/HIV (n = 30) infection upon stimulation with any HBV protein (core, env and pol)-derived peptide pools. Only values > 0 are shown. Median values and interquartile (25. and 75. percentile) range are indicated. (Mann-Whitney U test, ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$)

Median frequencies of IFN-γ producing HBV-reactive CD3 T cells were significantly higher ($p = 0.0479$) in patients with t-HBV (0.09 %, range 0.003 – 0.42 %) than with HBV/HIV (0.03 %, range 0.001 – 0.53 %) infection. IL-2 production was similar in HBV-reactive CD3 T cells of patients with t-HBV (0.03 %, range 0.001 – 0.22 %) or HBV/HIV (0.02 %, range 0.001 – 0.07 %) infection, while TNF producing CD3 T cells were slightly lower in patients with t-HBV (0.05 %, range 0.005 – 4.37 %) than with HBV/HIV (0.07 %, range 0.009 – 0.97 %) infection.

Overall, whereas neither frequencies of HBV-reactive, nor HBV antigen-reactive CD3 T cells were different, the IFN-γ production was significantly higher in patients with t-HBV than with HBV/HIV infection.

## 2.4 Peripheral HBV-reactive T cells do not correlate with the staging of HIV/AIDS and hepatitis B-related immunopathogenesis

As indicated before, the scope of cytokine production and the cytokine expression profile of antigen-reactive T cells can be different due to the state of the infection and the baseline immune status. Clinical data might correlate with the frequency of reactive T cells. The influence of HBV or HIV co-infection on parameters that are defining for the stages of HIV/AIDS and HBV-related immunopathogenesis is therefore investigated here.

CD4 T-cell counts are a main indicator for the staging of HIV infections and low levels are an AIDS-defining parameter, but CD4 T cells are also essential for functional antiviral immunity. HBV core-, env- and pol-reactive CD8 T-cell frequencies of patients with HBV/HIV infection were color-coded according to their peripheral CD4 counts. Frequencies of reactive CD8 T cells upon stimulation with the comparative antigens HIV nef and FEC are shown (Figure 36).

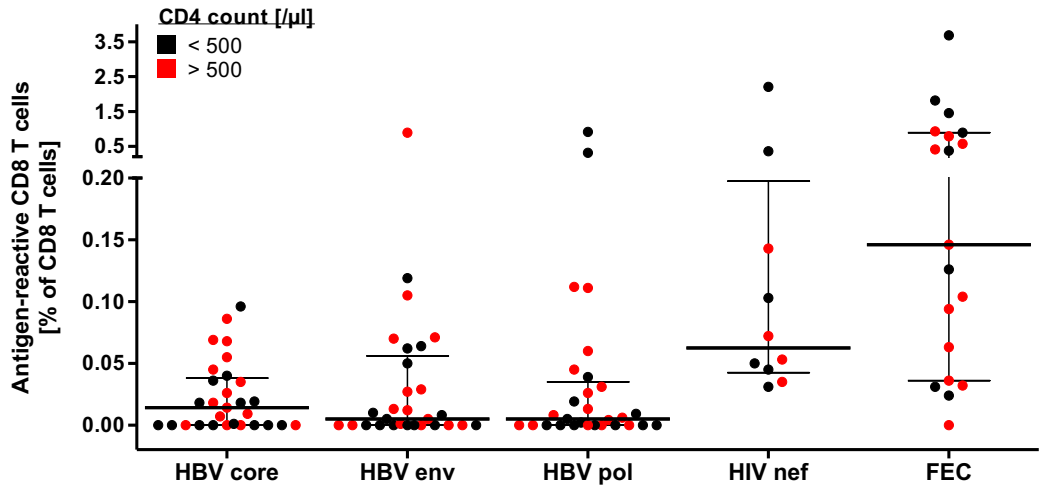


Figure 36: **No clustering of antigen-reactive CD8 T cells according to the CD4 T-cell counts.** Frequencies of CD8 T-cell responses of patients with HBV/HIV infection were determined by flow cytometry-based ICS after stimulation with an HBV core- (n = 30), env- (n = 30) and pol- (n = 30), HIV nef-derived (n = 10) or FEC-based (n = 19) peptide pool. High (> 500 CD4 cells/µL) CD4 counts are indicated in red and low (< 500 CD4 cells/µL) CD4 counts in black. Median values and interquartile (25. and 75. percentile) range are indicated.

No clustering of the frequency of antigen-reactive CD8 T cells with high (> 500 CD4 cells/µL) or low (< 500 CD4 cells/µL) CD4 counts was observed in any stimulation. Similar distributions of high and low CD4 T-cell counts were found for high, low or non-responders of HBV-reactive T cells.

Progressive hepatitis B causes damage to the liver, which can be detected by rising liver-specific enzymes in the peripheral blood. Serum liver enzyme AST and ALT levels therefore indicate the extent of damage to the organ [463].

Median AST levels were significantly higher ($p = 0.0026$) in patients with t-HBV (35.5, range 26 – 77 U/L) than with HBV/HIV (25, range 15 – 82 U/L) infection. Significantly higher ALT levels ($p = 0.0038$) were found in the serum of patients with t-HBV (43.5, range 19 – 106 U/L) in comparison to patients with HBV/HIV (28.5, range 11 – 96 U/L) infection (Supplemental Figure 1).

These indications of higher liver damage in patients with t-HBV infection were analyzed by correlating the frequency of peripheral env-reactive CD8 T cells with plasma AST and ALT levels (Figure 37, page 90).

Frequencies of HBV env-reactive CD8 T cells showed a non-significant, weak correlation with ALT levels of patients with t-HBV ($r^2 = –0.092$) or HBV/HIV ($r^2 = 0.081$) infection. Specifically, the frequency of HBV env-reactive CD8 T cells correlated negatively with rising ALT levels for patients with t-HBV and positively with HBV/HIV infection. This was similar for AST levels with which HBV env-reactive CD8 T cells correlated weakly, non-significantly for patients with t-HBV ($r^2 = –0.045$) and HBV/HIV ($r^2 = 0.126$) infection.

Although significant differences in the ALT and AST serum liver enzyme levels between the cohorts of patients with HBV/HIV or HIV infection were observed (Supplemental Figure 1), these did not correlate with the frequency of peripheral HIV nef-reactive CD8 T cells. Specifically, HIV nef-reactive T cells revealed a non-significant, weak correlation with plasma ALT data for patients with HIV ($r^2 = 0.082$), but not HBV/HIV ($r^2 = -0.005$) infection. Here, HIV nef-reactive CD8 T cells correlated positively with rising ALT levels for patients with HIV

infection. HIV nef-reactive CD8 T cells correlated strongly ($r^2$ = 0.642) and significantly ($p$ = 0.0053) with rising AST levels for patients with HIV, but not with HBV/HIV ($r^2$ = 0.008) infection.

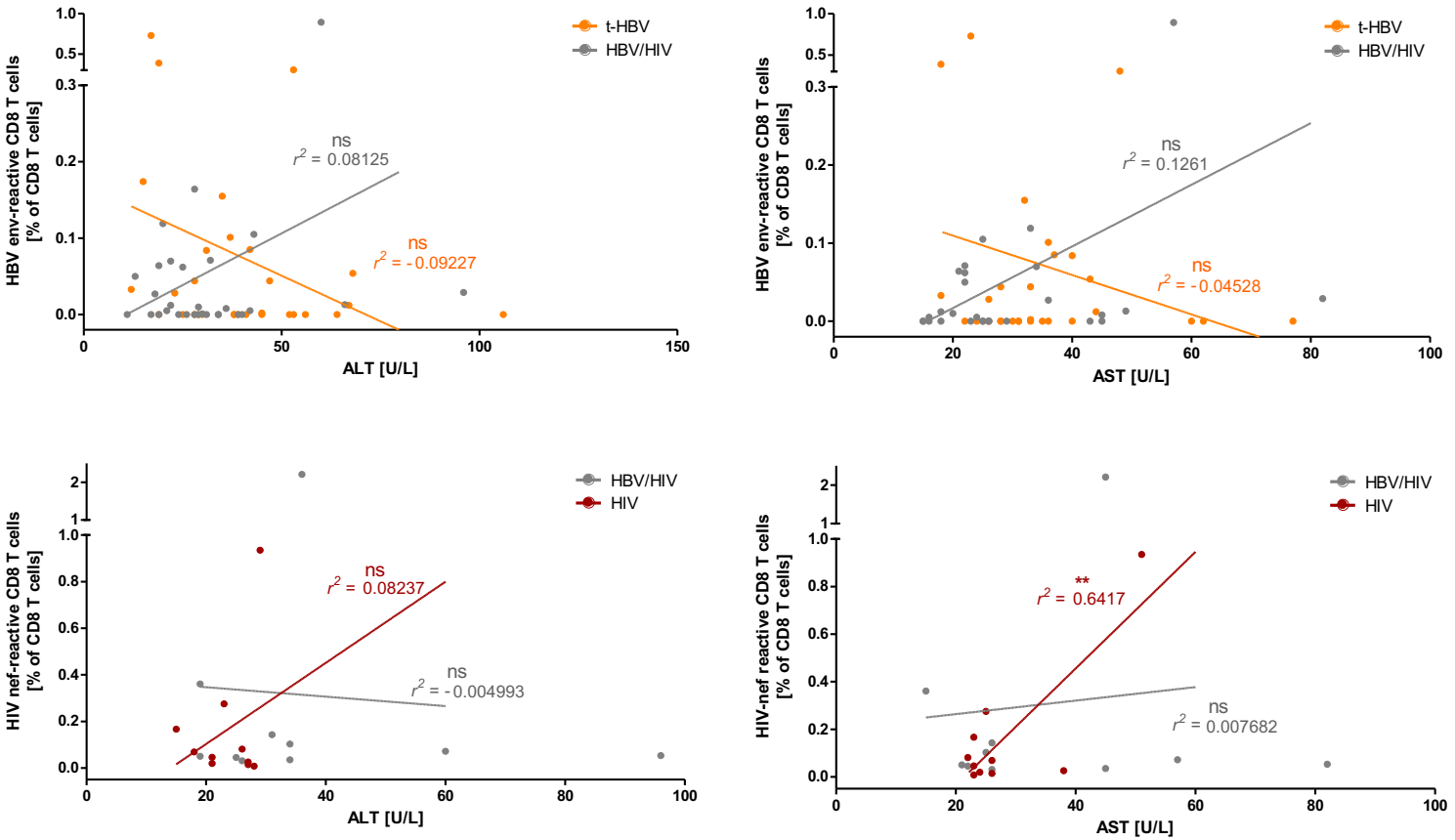


Figure 37: **Correlation of HBV- and HIV-reactive CD8 T cells with ALT and AST levels.** Serum liver enzyme levels of ALT and AST are correlated with reactive CD8 T cells of patients with t-HBV (n = 20), HBV/HIV (n = 30 for the first and n = 10 for the second stimulation) or HIV (n = 10) infection after stimulation with an HBV env- or an HIV nef-derived peptide pool. (Spearman correlation, ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$; correlation coefficient $r^2$ equates to the decimal of the slope with: no correlation $|r^2| < 0.01$, weak correlation $|r^2| \geq 0.01$, moderate correlation $|r^2| \geq 0.2$, strong correlation $|r^2| \geq 0.4$)

### 2.5 Regulatory T-cell frequencies are similar in t-HBV and HBV/HIV patients

Regulatory T cells can suppress cytotoxic T cells e.g. by the release of anti-inflammatory cytokine IL-10 and are mainly identified by their expression of the surface markers CD4 and CD25, as well as the transcription factor FoxP3. In HBV/HIV co-infection, regulatory T cells suppress the cytokine release of HBV-specific T cells in a PD-1 dependent manner that could be reversed by HBV-active ART [455].

Frequencies of $CD25^+$ $FoxP3^+$ regulatory CD4 T cells are depicted for patients with t-HBV, HBV/HIV, HIV/rHBV or HIV infection as well as HBV/HIV naïve subjects (Figure 38). The cohort with t-HBV infection had higher, but not significantly different, levels of regulatory T cells (3.82 %, range 2.21 – 5.79 %) than patients with HBV/HIV infection (2.19 %, range 1.07 – 4.66 %). HBV/HIV naïve subjects had comparable Treg frequencies (2.22 %, range 0.91 – 3.47 %) to patients with HBV/HIV, but significantly ($p$ = 0.0068) lower levels than patients with t-HBV infection.

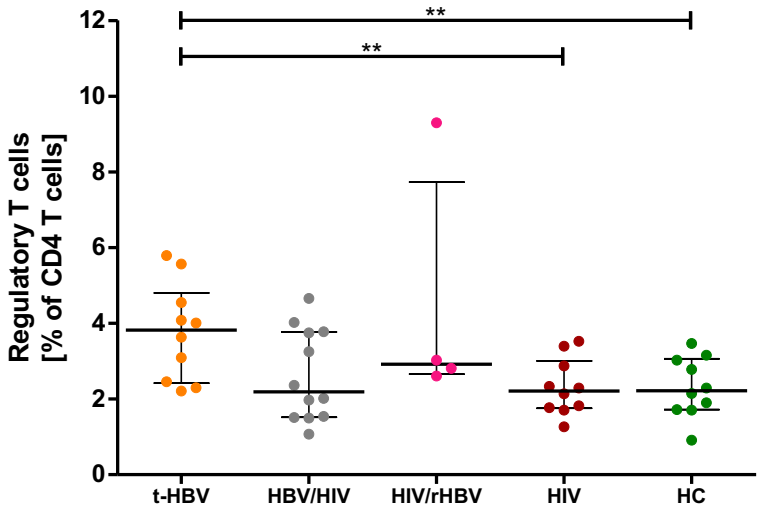


Figure 38: **Frequency of regulatory T cells.** Frequencies of $CD25^+$ $FoxP3^+$ regulatory CD4 T cells were determined by flow cytometry. Shown are frequencies of CD4 Treg of patients with t-HBV (n = 10), HBV/HIV (n = 12), HIV/rHBV (n = 4) and HIV (n = 10) infection, as well as HBV/HIV naïve subjects (HC; n = 10). Median values and interquartile (25. and 75. percentile) range are indicated. (Primary Kruskall-Wallis test (ANOVA) with secondary Mann-Whitney U test, ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$)

## 2.6 T-cell exhaustion is more prominent in HBV/HIV co-infection

T-cell exhaustion is a prominent mechanism of predominately CD8 T-cell dysfunction in persistent viral infection and chronic disease. Gradual loss of cytokine expression is one of the functional observations in T-cell exhaustion.

Certain markers like PD-1, CD39 and TIGIT are found on the surface of exhausted T cells, but there are also transcription factors like T-bet, Eomes and Tcf-1 that are differentially expressed (or absent) in exhausted T cells. Taking these factors together, it is possible to further describe the phenotype of antigen-experienced (non CD45RA$^{+}$ CCR7$^{+}$) CD8 T cells according to the expression of CD127$^{+/-}$ and PD-1$^{high/low/-}$ and evaluate their levels of the other exhaustion markers.

Since differences in the production of single cytokines and the cytokine expression profile of CD8 T cells of patients with t-HBV or HBV/HIV infection were observed, an assessment of the presence of phenotypes of exhausted T cells was performed. The expression of the aforementioned markers on antigen-experienced CD8 T cells was therefore compared in the different cohorts (Figure 39).

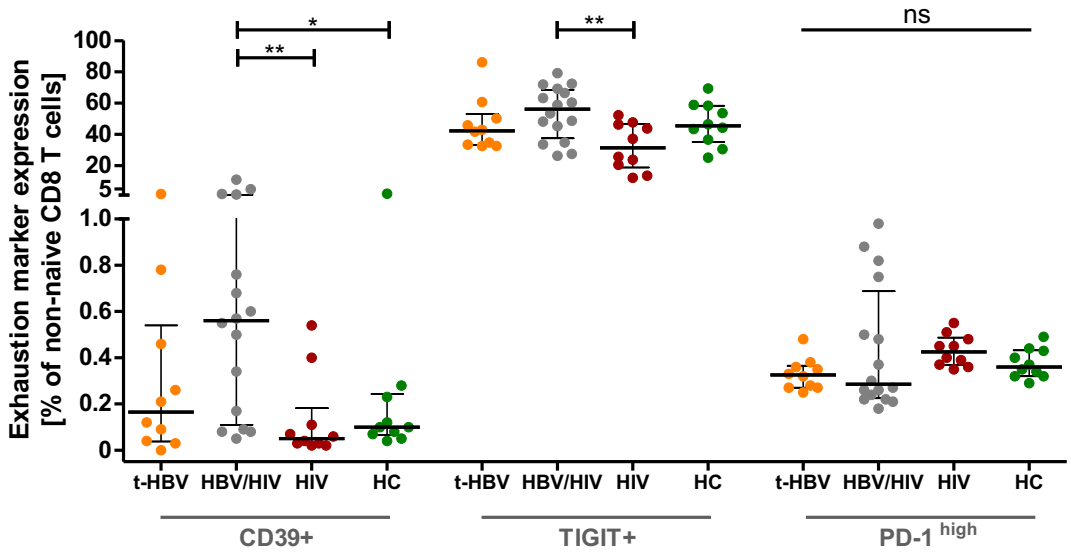


Figure 39: **Expression of exhaustion markers by antigen-experienced CD8 T cells.** The expression of the T-cell exhaustion markers CD39, TIGIT and PD-1$^{+}$ on antigen-experienced (non CD45RA$^{-}$ CCR7$^{-}$) CD8 T cells of patients with t-HBV (n = 10), HBV/HIV (n = 16) and HIV (n = 10) infection and HBV/HIV naive subjects (HC; n = 10) was determined by flow cytometry. Median values and interquartile (25. and 75. percentile) range are indicated. (Primary Kruskall-Wallis test (ANOVA) with secondary Mann-Whitney U test, ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$)

Two groups expressing different levels of CD39 were observed. Most patients were in the first group, which had 0 – 1 % of $CD39^+$ CD8 T cells, whereas a second group of 6 patients (HC: n = 1, HBV: n = 1, HBV/HIV n = 4, HIV: n = 0) had frequencies from 1 – 10 %. The median level of $CD39^+$ CD8 T cells was higher in patients with HBV/HIV (0.56 %, range 0.05 – 10.97 %) than with t-HBV (0.17 %, range 0.00 – 1.73 %) infection. Frequencies of $CD39^+$ CD8 T cells were similar in t-HBV and HBV/HIV naïve subjects (0.10 %, range 0.04 – 2.01 %), but patients with HBV/HIV infection had significantly higher frequencies ($p = 0.0348$) than HBV/HIV naïve subjects.

TIGIT levels were generally higher than CD39 levels. Patients with HBV/HIV infection (56.12 %, range 26.14 – 79.18 %) had higher median TIGIT levels than patients with t-HBV infection (42.19 %, range 32.48 – 86.14 %). Both were comparable to frequencies of $TIGIT^+$ CD8 T cells of HBV/HIV naïve subjects (45.39 %, range 24.96 – 69.36 %).

$PD\text{-}1^{high}$ levels associated with T-cell exhaustion were evaluated. The median $PD\text{-}1^{high}$ expression was quite low. Patients with t-HBV (0.33 %, range 0.25 – 0.48 %) or HBV/HIV (0.29 %, range 0.18 – 0.98 %) infection had comparable frequencies. $PD\text{-}1^{high}$ levels of CD8 T cells were similar to HBV/HIV naïve individuals (0.36 %, range 0.29 – 0.49 %). The frequencies of $PD\text{-}1^{high}$ antigen-experienced CD8 T cells were not significantly different in the compared cohorts.

To determine the T-cell phenotype in detail, approaches that evaluate the expression of multiple markers on every cell deliver more information than the analysis of the expression rates of singular exhaustion markers. Machine-learning and clustering algorithms like the SPADE tree and tSNE algorithms can visualize complex multi-dimensional data as shown below.

The SPADE tree analysis showed clusters of cells that were grouped according to their PD-1 and CD127 expression (Figure 40). Four distinct groups of cell clusters with high or low PD-1 and CD127 expression were found. Overall expression of the transcription factor Eomes is quite low over the whole SPADE tree and cell clusters with higher expression are mostly found in PD-1$^{+}$ CD127$^{+/-}$ T cells. Evaluating the SPADE tree showing the expression of T-bet in the different cell clusters revealed overall high levels with only few cell clusters showing a low expression, which are mostly found in CD127$^{+}$ cells, especially CD127$^{+}$ PD-1$^{+}$ CD8 T cells.

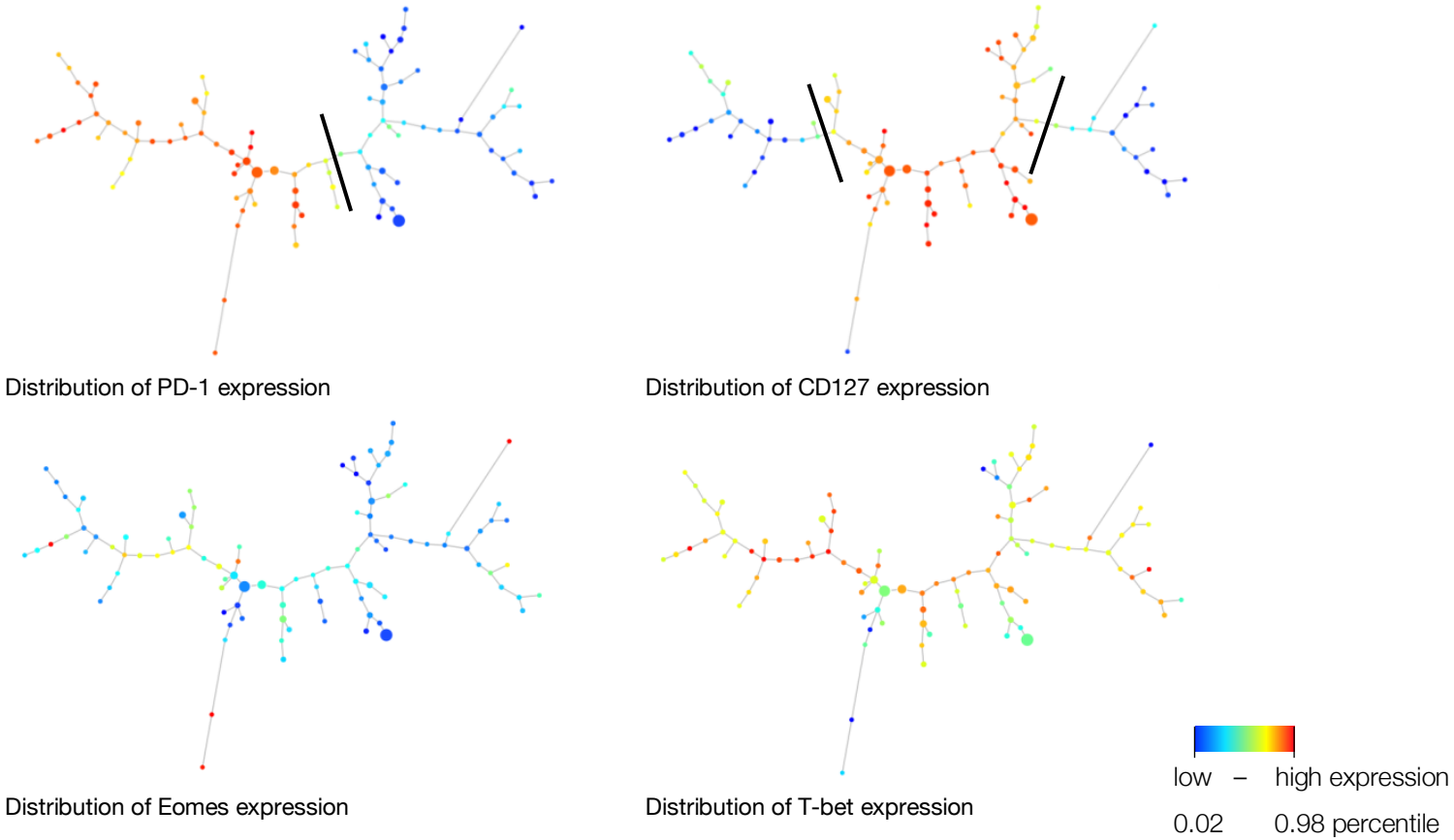


Figure 40: **SPADE tree analysis of exhaustion marker expression.** Exhaustion marker expression was categorized and visualized with the SPADE algorithm. Each knot represents a cluster of antigen-experienced CD8 T cells that contains cells with similar phenotype. Branches are indicating relations of T cells with each other. Low expression of markers is indicated with blue colors, while green and yellow show intermediate and orange shows high expression of the marker in the cluster of T cells. CD8 T-cell clusters were categorized by their PD-1 and CD127 expression. The expression of transcription factors Eomes and T-bet in the cell clusters was evaluated according to the expression of PD-1 and CD127.

SPADE tree analysis showed distinct expression patterns of the transcription factors T-bet and Eomes over the range of cell clusters that were clustered by the SPADE tree algorithm. CD8 T-cell subsets can be identified according to their T-bet and Eomes expression with T-bet$^{high}$ (Eomes$^{+/-}$) cells being functionally active, while T-bet$^{low}$ Eomes$^{+}$ CD8 T cells are functionally impaired [464].

Frequencies of antigen-experienced (non CD45RA$^{+}$ CCR7$^{+}$) CD8 T cells expressing T-bet and/or Eomes were determined (Figure 41).

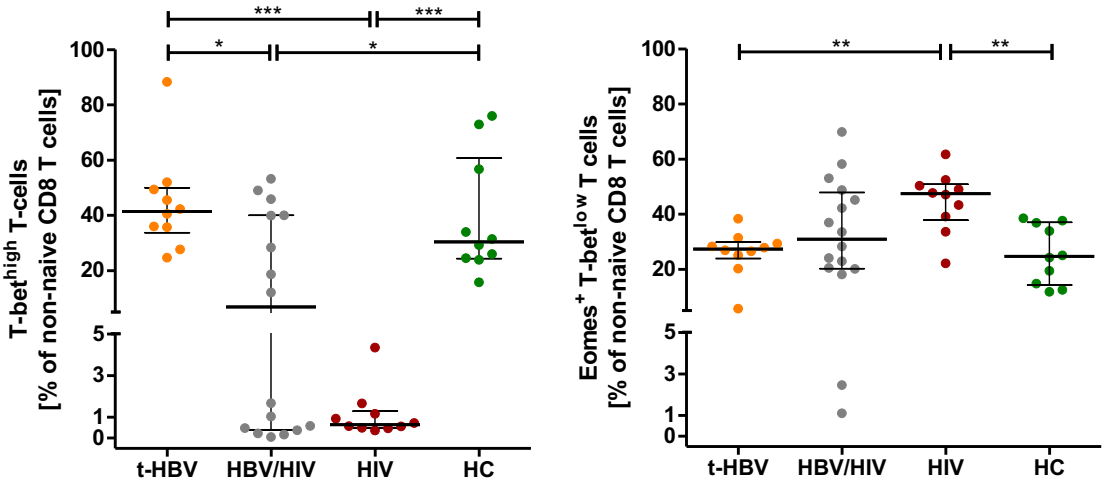


Figure 41: **Expression of T-cell functionality defining transcription factors related to T-cell exhaustion.** Frequencies of antigen-experienced (non CD45RA$^{+}$ CCR7$^{+}$) CD8 T cells producing transcription factors that are related to T-cell function (T-bet$^{high}$ Eomes$^{+/-}$) and dysfunction (T-bet$^{low}$ Eomes$^{+}$) were analyzed by flow cytometry for patients with t-HBV (n = 10), HBV/HIV (n = 16) or HIV (n = 10) infection and HBV/HIV naive subjects (HC; n = 10). Median values and interquartile (25. and 75. percentile) range are indicated. (Primary Kruskall-Wallis test (ANOVA) with secondary Mann-Whitney U test, ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$)

Three groups of patients expressing different ranges of T-bet$^{high}$ levels were found in the cohorts. The first group had less than 5 % of T-bet$^{high}$ CD8 T cells, the second had 10 – 60 % and lastly very few patients were deviating with 60 – 90 % of T-bet$^{high}$ CD8 T cells. Only one patient with t-HBV infection (median 41.45 %, range 24.75 – 88.36 %) had more than 60 % T-bet$^{high}$ CD8 T cells with every other patient showing 10 – 60 %. Half of the patients with HBV/HIV infection (median 6.88 %, range 0.05 – 53.26 %) revealed to have low levels of less than 10 % T-bet$^{high}$ CD8 T cells, whereas the other 50 % of patients had frequencies of 10 – 60 %. By contrast, patients with HIV infection (median 0.65 %, range

0.37 – 4.34 %) only had low levels of less than 10 % T-bet$^{high}$ CD8 T cells. Similarly, HBV/HIV naïve individuals had mostly 10 – 60 % (median 30.41 %, range 15.79 – 75.99 %), but two subjects had more than 60 % T-bet$^{high}$ CD8 T cells.

Overall, patients with HBV/HIV infection revealed both, the high and the low group of T-bet$^{high}$ expression, while patients with t-HBV infection only had the high levels and patients with HIV infection only low levels in antigen-experienced CD8 T cells. The frequencies of T-bet$^{high}$ CD8 T cells were significantly ($p$ = 0.0143) higher in patients with t-HBV than with HBV/HIV infection. Frequencies of the T-bet$^{high}$ CD8 T-cell subset of patients with HBV/HIV ($p$ = 0.0374), but not t-HBV infection were significantly higher in comparison to HBV/HIV naïve subjects.

The median frequencies of the subset of Eomes$^{+}$ T-bet$^{low}$ CD8 T cells were similar in patients with t-HBV (27.36 %, range 5.78 – 38.41 %) or HBV/HIV (30.91 %, range 1.11 – 69.85 %) infection, but data from both cohorts were also comparable to Eomes$^{+}$ T-bet$^{low}$ CD8 T-cell frequencies of HBV/HIV naïve individuals (24.69 %, range 11.85 – 38.53 %).

Data so far showed that patients with t-HBV or HBV/HIV infection had significantly different levels of functional (T-bet$^{high}$), but not of dysfunctional (T-bet$^{low}$ Eomes$^{+}$) CD8 T-cell subsets (as shown in Figure 41).

The tSNE algorithm was used to cluster CD8 T cells from patients with t-HBV, HBV/HIV or HIV infection and HBV/HIV naive subjects according to their T-cell exhaustion marker expression and visualize the multi-dimensional information (Figure 42). The production of exhaustion markers PD-1, CD39 and TIGIT and the surface marker CD127, as well as the transcription factors T-bet, Eomes and Tcf-1 was analyzed in dependency of each other. The tSNE algorithm gives the option to perform the analysis in a multi-dimensional way that reduces the information to a 2D blot in order to visualize the relation.

The algorithm revealed shared CD8 T-cell clusters in antigen-experienced CD8 T cells of patients with t-HBV, HBV/HIV or HIV infection and HBV/HIV naive subjects. When comparing CD8 T-cell subset clustering visualized according to their exhaustion marker expression, T cells of patients with t-HBV or HIV infection showed mostly distinct clusters, while T cells of patients with HBV/HIV infection had some distinct, but also shared clusters. HBV/HIV naïve subjects revealed CD8 T cells that were distributed widely, but also showed distinct clusters, which were partially shared with data from patients with HIV infection.

Distinct clusters of terminally exhausted T cells (PD-1$^{high}$ CD127$^{-}$ Tcf-1$^{-}$) and memory-like T cells (PD-1$^{+}$ CD127$^{+}$ Tcf-1$^{+}$) were found (Figure 42 G-H). A comparison of the incidence of these T-cell subsets in the different cohorts revealed the cluster of terminally exhausted CD8 T cells to be more prevalent in patients with HBV/HIV than with t-HBV or HIV infection and than in HBV/HIV naïve subjects. The cluster of memory-like T cells was a lot more prevalent in patients with t-HBV and HBV/HIV than with HIV infection or in HBV/HIV naïve subjects.

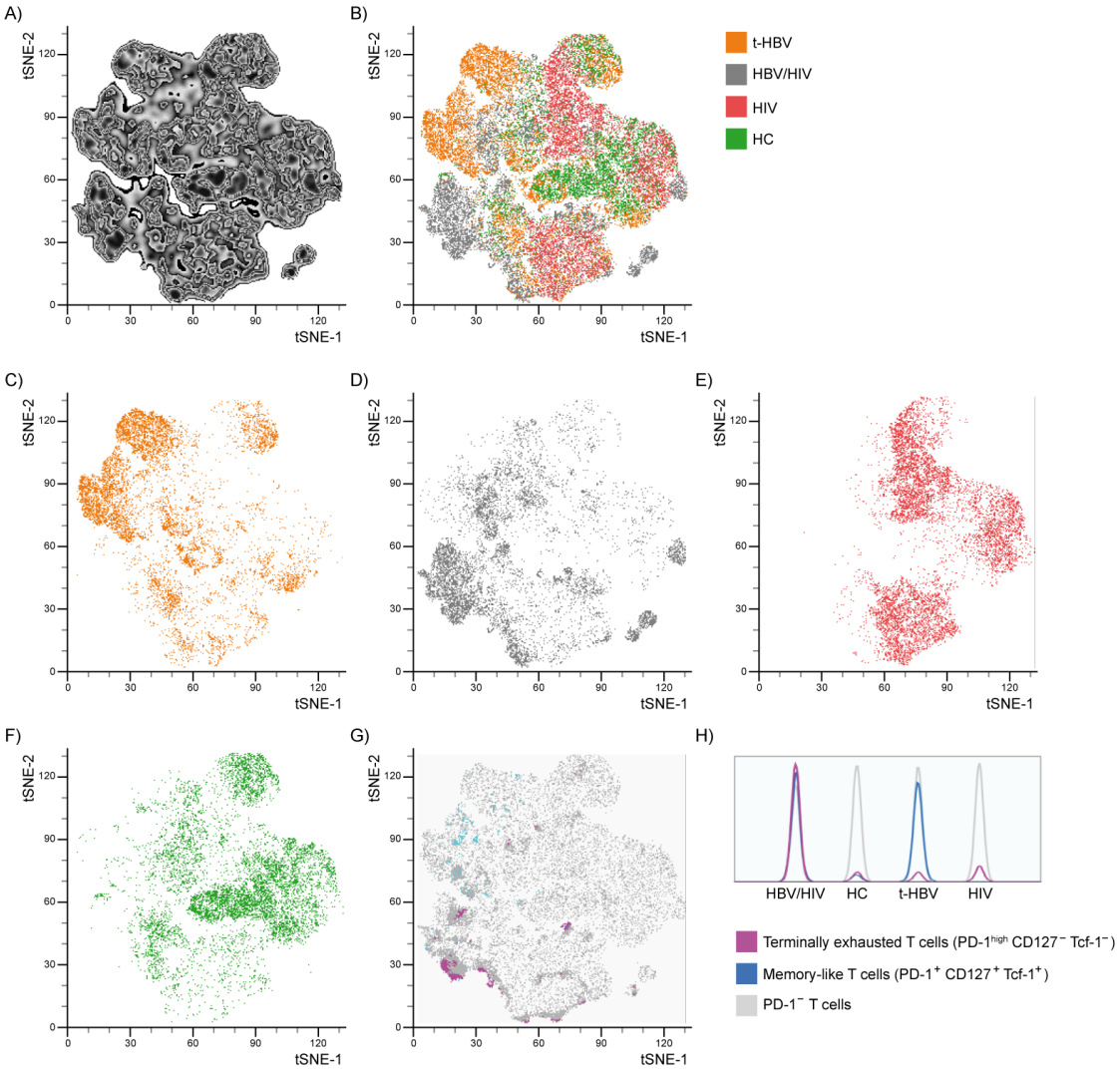


Figure 42: **tSNE analysis of exhaustion marker expression.** The tSNE algorithm was used to create a multi-dimensional visualization of T-cell clusters dependent on their expression of the surface markers PD-1, CD39, TIGIT, CD127 and the transcription factors Eomes, T-bet and Tcf-1. Data from patients with t-HBV, HBV/HIV or HIV infection and HBV/HIV naïve individuals was included. A) The tSNE algorithm led to a clustering of T cells. B)-F) Similarly, T cells of the cohorts of patients with t-HBV (yellow), HBV/HIV (grey)and HIV (red) infection and HBV/HIV naïve/HC (green) subjects are shown according to their clustering upon tSNE analysis. G) Occurrence of terminally exhausted T cells (pink; PD-1$^{high}$ CD127$^{-}$ Tcf-1$^{-}$), memory-like T cells (blue; PD-1$^{+}$ CD127$^{+}$ Tcf-1$^{+}$) and PD-1$^{-}$ T cells in the multi-dimensional tSNE algorithmic presentation showed distinct clusters. H) Relative portion of these T-cell phenotypes is presented for the cohorts of patients with t-HBV, HBV/HIV or HIV infection as well as HBV/HIV naïve individuals.

# 3. Discussion

## 3.1 Impact of HIV on the course of HBV infection

2.7 million people have an active HBV/HIV co-infection, and 70 – 90 % of all HIV-infected individuals show evidence of an active or past HBV infection [1, 390]. The natural history of hepatitis B is mainly determined by the breadth and activity of HBV-specific T cells [3, 6]. The effect of progressive HIV/AIDS, which is linked to T-cell depletion and immunopathogenesis, was already assessed in the context of HBV/HIV co-infection showing inconclusive results regarding an unchanged [399] or decreased [456] HBV-specific T-cell immunity.

Identification of T-cell populations, antigen-specificity and involvement of regulatory mechanisms that are modulated upon HIV co-infection in comparison to HBV mono-infection could not only allow better understanding of the pathogenesis of HBV infection, but also provide insights into key immunological factors involved in HBV persistence and clearance.

Although higher numbers of HBV/HIV co-infected patients in comparison to HBV mono-infected subjects spontaneously clear acute [465] and persistent HBV infection (HBV mono: 1 – 3 % in [430], 2 % in [151], 1 % in [466] versus HBV/HIV: 15 % in [467], 7 % in [468], 7 % in [429], 18 % in [469], 18 – 20 % in [428], 13 % in [470], 8 – 36 % in [427], 4 % in [471], 8 % in [472]) and HBV-active ART is available, which suppresses but does not cure HIV infections, mortality is high in HIV co-infection [411, 412, 415-417] with long-term complications [416, 417, 473] and progression to AIDS [411] and liver cirrhosis with possible HCC [474, 475].

## 3.2 Impact of HIV on the baseline immune/T-cell activation

In this cross-sectional study, a PBMC biobank of HBV mono-, HBV/HIV co- and HIV mono-infected patients was established to characterize the baseline immune status, T-cell activation and CMI (cell-mediated immunity) in HBV mono- and HBV/HIV co-infection.

Patients were stratified according to their disease course to achieve more homogenous cohorts than in previous studies characterizing non-homogeneous cohorts [399, 456].

### 3.2.1 Socio-demographic and clinical characteristics

Assessment of the socio-demographic and clinical backgrounds of the patients is performed to identify factors that could influence the analyzed T-cell immunity. The cohorts of HBV mono- and HBV/HIV co-infected patients were quite similar regarding socio-demographic factors like sex distribution and age. All patients were under antiviral therapy. The HBV/HIV co-infected patient's median CD4 count is lower than the reference value (800 CD4 cells/µL, range 500 – 1500 cells/µL) of healthy individuals. A comparison of the CD4 counts in HBV/HIV co-infected patients and HIV mono-infected subjects showed similar values implying that the HBV co-infection has no detrimental effect on the CD4 counts [476], while the comparison of CD4 counts with the CD4 frequency revealed a significant correlation, underlining that PBMC isolation and stimulation in assays to determine the T-cell phenotype and reactivity preserved the CD4 T cells. The CD4 to CD8 ratio is skewed in HBV/HIV co-infection with much lower CD4 cells than the healthy ratio of 58 % : 33 %, which is typical in HIV infection [477] and indicates a grave difference in the number of the CD4 helper and cytotoxic CD8 T cells between both cohorts. This could affect CD4 T helper cell-dependent CD8 T-cell priming, as well as the development of antibody-secreting plasma cells [9]. The origin of HBV mono- and HBV/HIV co-infected patients was quite similar with only patients from African origin being strikingly more frequent in HIV co-infection, and t-HBV patients having a quite high share from the Western Pacific Region [1]. The origin might also correlate with the mode of transition [478-480] and the HBV genotype the patients are infected with [1], which can influence the recognition of the antigen and also the strength of the T-cell response [481]. The main HBV genotypes in the European region are genotypes A and D, genotypes A and E in Sub-Saharan Africa and genotypes B and C in the Western Pacific region [146].

The socio-demographic characteristics between HBV mono- and HBV/HIV co-infected patients were quite similar. Differences in their general and HBV-specific T-cell immunity could therefore not be caused by these factors. Only the origin differed meaningfully, which affects the HBV genotype as discussed earlier and influences at which age and which mode of transmission the patients are primarily infected with HBV.

While the HIV RNA and HBV DNA loads of HBV co- and HBV mono-infected individuals were suppressed under therapy, the HBsAg levels were 2-fold higher in HBV mono-infected patients, which might be causative for T-cell exhaustion [482]. Previous studies showed a similar picture with higher HBV DNA loads in HBV mono- than HBV/HIV co-infected patients [402, 483]. They, nevertheless, showed higher HBeAg seroconversion rates in co-infected cohorts, whereas this was not observed in the present study [402, 483]. Although the median serum enzyme liver levels ALT and AST were also high in HBV mono-infected, only the values of some patients were not in the range of healthy subjects, which could indicate liver damage due to virus clearance [484]. This was also described before, but is also linked to fatty liver disease and immune reconstitution disease (as described in 1.3.2) [419, 485, 486]. The HIV RNA load was lower in HIV mono- than in HBV/HIV co-infected patients indicating a better control of the infection or a higher therapy success [487]. By contrast, it was previously reported that there was no significant difference in HIV RNA loads between HIV mono- and HBV/HIV co-infected patients [488].

### 3.2.2 Baseline immune and activation status

$CD3^+$ lymphocytes represent the group of T cells in leukocytes and therefore play the central cellular role in the adaptive immune system [9]. A functional and stable or dysfunctional and scarce T-cell immunity determines if acute HBV infections resolve or persist [6]. The number of CD4 and CD8 T cells may therefore influence the magnitude of detected HBV-specific T-cell responses by missing T helper function or absence of cytotoxic or memory T cells [9]. This was also shown in the context of HBV vaccination, which is majorly influenced by the CD4 and CD8 frequencies [489]. CD3 T-cell frequencies were significantly lower in HBV mono- than HBV/HIV co-infected patients in this study. Others have shown similar CD3 frequencies in HBV mono and HBV/HIV co-infected patients [476]. While the CD4 and CD8 T-cell frequencies were significantly different between the cohorts of HBV mono-infected patients and HBV/HIV co-infected patients in this study, T-cell populations like antigen-experienced CD4 T cells are known to be lost in HIV-infected cohorts [459, 477, 490] or would rather be migrated to the liver [491]. Lower CD4 T-cell frequencies of HBV mono-infected patients in contrast to healthy controls were reported elsewhere (51 % vs. 63 %, [492]), whereas they do not reach the low CD4 T-cell frequency of HBV/HIV co-infected

patients (31 %, [493]). Maturation of CD8 T cells was also shown to be skewed in viral infections [494]. These results might again indicate differences in the CD4 helper and cytotoxic CD8 T cells numbers between both cohorts [9], which could affect the HBV-specific T-cell immunity.

In this study, no significant difference regarding the frequency of peripheral CD8 T-cell subsets between HBV mono- and HBV/HIV co-infected patients were found. While both cohorts show lower levels of naïve T cells than HBV/HIV naïve subjects, the differences are not significant. There seems to be a trend of slightly higher levels of effector memory and terminally differentiated effector memory CD8 T cells in HBV/HIV co-infection. This might show a higher priming rate of T cells in the co-infection, which is observed in HIV patients, whereas *in vitro* data showed HIV-related reduced T-cell priming by DC [495], but does not fit to the idea that HIV infection reduces antigen presentation by infecting APC and the downregulation of HLA [496, 497]. It was show that changes in the T-cell subset ratios due to increased priming and activation are mainly induced locally, where high amounts of antigen are present and would therefore in the context of HBV-specific T cells be expected in the liver rather than the periphery [9, 498-501].

It was reported that presence of antigen leads to activation of T cells, which release cytokines inducing ongoing inflammation and thereby pathogenesis [502-504]. Analyzing the activation status of T cells, activation markers PD-1 and CD25 on CD4 and CD8 T cells were determined. There was no significant difference between HBV mono- and HBV/HIV co-infected patients regarding the production of the marker PD-1, but higher levels than in HBV/HIV naïve subjects for both CD4 as well as CD8 T cells. Others reported higher PD-1 levels in HBV mono- than in HBV/HIV co-infection, which was assessed in similar patient cohorts as were included in this study, whereas data was collected from serum with ELISA and not on T cells with flow cytometry [505]. Also data from HIV mono-infected patients in this study showed no significantly higher activation, but it is stated that T cells of virus-infected individuals are more strongly activated [9], which goes in line with data showing HIV to promote T-cell activation more strongly than other infections [506-512].

The expression of CD25 on CD8 T cells also showed no significant difference between HBV mono- and HBV/HIV co-infection, but significantly higher levels in in comparison to HBV/HIV naïve subjects, but no difference between the virus-infected cohorts. By contrast, CD4

T cells of HBV mono-infected patients were found to have significantly lower levels of CD25 than of HBV/HIV co-infected subjects, while both were not significantly lower than HBV/HIV naïve subjects. This goes in direct contrast to previous data that showed low CD25 levels on CD4 T cells in HIV infection [513]. This is not only a sign for T-cell activation, but might also be a hint regarding regulatory T cells, since Tregs are $CD25^+$ [514]. Data on regulatory T-cell frequencies of patients with HBV/HIV co-infection is shown in Figure 38, while the analysis is presented in part 2.5.

Overall, no significant differences in CD4 or CD8 T-cell activation was found in HBV mono- and HBV/HIV co-infected patients in this study.

### 3.3 Impact of HIV on the HBV-specific cellular-mediated immunity

Frequency of HBV-specific T cells, their phenotype and cytokine production were characterized with flow cytometry-based intracellular cytokine staining to evaluate whether and to which extent HIV co-infection leads to an impaired HBV-specific cell-mediated immunity. Distinct cytokine expression profiles of CD4 and CD8 T cells upon stimulation with HBV protein-derived peptide pools were found to be significantly different in patients with HBV mono- and HBV/HIV co-infection as shown in Figure 22 and 29.

While a few studies were already conducted to analyze the state of HBV-specific immunity in HBV/HIV co-infection, the extent to which HIV co-infection modulates HBV-specific T-cell immunity remains elusive or controversial as elaborated below.

While differences of the cytokine expression profile of HBV antigen-specific CD4 and CD8 T cells in HBV/HIV co-infection in comparison to the HBV mono-infection were found in this study, there was no significant difference in the frequency of HBV core, env or pol reactive CD4 or CD8 T cells.

Similar results were also shown in a study by Chang et al., who reported no significant differences in the HBV-specific CD8 T-cell frequencies, but in their cytokine expression profile of HBV mono- and HBV/HIV co-infection. Readouts were performed with flow cytometry-based cytokine staining (IFN-γ, TNF) of whole blood after stimulation with overlapping peptides spanning the entire HBV genome. Although cohort sizes were similar to our study (Table 2) with 24 HBV/HIV and 39 HBV patients, only treatment-naïve subjects with Asian origin were included contrary to the present study [399].

By contrast, Zhang et al. reported (non-significantly) lower HBV core-specific T-cell responses in HBV/HIV co- than HBV mono-infected patients. The study compared 25 HBV infected patients to a longitudinal analysis of 11 HBV/HIV co-infected patients differentiated by their HBeAg status. They report ELISPOT assay-based data after stimulation of PBMC with recombinant HBV env and core proteins. Differentiation of CD4 and CD8 T cells was not performed, while recombinant proteins mainly stimulate CD4 T cells, also CD8 T cells could contribute to the results due to cross-presentation [456].

Similarly, Ren et al. showed significantly lower IFN-γ producing CD8 T cells in HBV/HIV co- than HBV mono-infection. This study compared 39 patients with chronic hepatitis B with

17 patients with chronic hepatitis B and HIV co-infection. Peptide pool with 28 peptides of 18 aa length, overlapping with 10 aa derived from the core protein and 4 published peptides (2x env, 1x core, 1x pol) were used for stimulation before flow cytometry-based ICS. A main difference to the present study is that the PBMC were expanded with IL-2, anti-CD28/CD49d and the HBV protein-derived peptide pools for 10 days [458].

Results in the present study deviated from the work of Chang et al. and Zhang et al. and Ren et al., which could arise from differences in the study design, the patient cohort and their socio-demographic background as well as the chosen immunoassays and antigenic stimulation approach. Comparability of cell-mediated immunity data from different studies could be improved by standardization of immunoassays.

Response rates are from here on defined as the ratio of individuals of the respective cohort, which show a positive cytokine release by T cells upon the indicated stimulation. HBV/HIV co-infected patients showed higher CD4 T-cell response rates upon virus-derived peptide pool stimulations than HBV mono-infected patients. HBV env-stimulation revealed 10 – 15 % lower response rates of CD4 T cells of HBV mono-infected patients. By contrast, CD4 T cells of HBV/HIV co-infected patients showed a 20 – 25 % lower response rate upon HBV pol-derived stimulation than other virus-derived stimulations resulting in a lower response rate than of HBV mono-infected patients. Median response rates of CD8 T cells of HBV mono-infected patients upon virus-derived peptide pool stimulation were higher than of HBV/HIV co-infected patients. There was a loss of 15 – 20 % of CD8 T-cell responsiveness upon HBV env-stimulation in HBV mono-infected patients in comparison to the other stimulations leading to lower response rates than HBV/HIV co-infected patients. Overall, there is a loss of response rates of CD4 and CD8 T cells upon HBV env-derived stimulation in HBV mono-infected patients in contrast to other virus-derived peptide pool stimulations. The group of Robert Thimme also already reported a reduced presence of HBV env-specific T cells [237]. On the contrary, it was reported before that HBV env and core are similarly immunogenic proteins [515], although HBc antibodies are found in nearly all HBV-infected individuals and HBsAg antibodies only in functionally cured patients [516, 517]. According to studies with mice, HBV core-specific T cells are found in the liver [518], which indicates the migration from the periphery. It remains elusive, if HIV infection inhibits the priming of T cells of a certain HBV antigen-specificity or mechanisms like T-cell dysfunction could decrease the response rates of HBV env-specific T cells, decreased

maturation of certain primed HBV antigen-specific T cells to their memory phenotypes, lower levels of HBV antigen-specific T cells in the periphery due to migration e.g. to the liver or loss of HBV antigen-specific T cells by apoptosis.

As an assay control, PBMC of different cohorts were stimulated with a comparative antigen peptide pool called FEC targeting epitopes of Flu, EBV and CMV [519, 520]. T cells of the patients reacted to the stimulation by cytokine production showing that the stimulation and flow cytometric detection worked. Additionally, the results show that T cells are re-activatable after cryopreservation of the PBMC as well as their activation mechanisms are in general not permanently inhibited by any HBV or HIV infection-related effects. When comparing the response rates, more HBV mono- and HBV/HIV co-infected patients had CD8, but no CD4 T-cell responses upon FEC peptide pool stimulation, which went in conjunction with the previous observation of higher FEC-reactive CD8 T-cell frequencies due to the design of the FEC peptide pool [519, 520]. Response rates upon any HBV-derived stimulation of CD8 T cells of both cohorts were lower than upon FEC stimulation. This can either be due to a lower priming rate of naïve T cells in HBV than in Flu, EBV and/or CMV infection or any reason discussed for differences in HBV antigen-specific T cells as well such as decreased maturation of primed HBV-specific T cells to their memory phenotypes, lower levels of HBV-specific T cells in the periphery due to migration e.g. to the liver, loss of HBV antigen-specific T cells by apoptosis or loss of responsiveness due to T-cell dysfunction e.g. T-cell exhaustion.

Total HBV-reactive and HBV core-, env- and pol- reactive CD4 T-cell frequencies, as well as the magnitude of single cytokine (IFN-γ, IL-2, TNF)-secreting HBV core-, env- and pol-reactive CD4 T cells were similar in patients with HBV mono- and HBV/HIV co-infection. While HIV infection leads to progressive loss of CD4 T cells [490], which was also observed in this work (Supplemental Figure 3), a quantitative loss of HBV-reactive CD4 T-cells was not found in HBV/HIV co-infection. While it is likely that HBV-specific T cells mainly stay in tissue that is not as affected by HIV infection-mediated loss of CD4 T cells, it is also possible that HBV-specific T cells are not infected by HIV and their apoptosis is therefore not specifically activated. Alternatively, the amount of newly primed HBV-specific CD4 T cells could be higher than the number of depleted HBV-specific CD4 T cells, due to successful antiviral treatment. Additionally, the frequency of virus-reactive CD4 T cells is influenced by their activation status. If HIV infection induces higher activation of HBV-specific T cells

leading to increased cytokine secretion, frequencies of HBV-reactive T cells were higher. While HIV-infection might induce a loss of HBV-specific T cells, which could therefore be lower than in HBV mono-infected patients, the frequency of reactive CD4 T cells in HBV/HIV co-infection could be higher and thereby show a similar frequency as in HBV mono-infection. Significantly higher activation marker levels on CD4 T cells were, nevertheless, not observed in HBV/HIV than in HBV patients. Alternatively, HBV infection-induced T-cell dysfunction could reduce the activation of HBV-specific CD4 T cells *in vivo* and thereby inhibit the infection of HBV-specific T cells and the progression of the HIV infection (as both entry and replication are dependent on T-cell activation [521]) and the loss of these T cells.

By contrast, the number of single T cells producing a certain number of cytokines in parallel, referred to as the functionality, was significantly higher in HBV core-reactive CD4 T cells of HBV than of HBV/HIV patients. The cytokine expression profile showed this in detail: trifunctional HBV core-reactive CD4 T cells were significantly more present in HBV mono- than in HBV/HIV co-infected patients. Nevertheless, HBV env-reactive CD4 T-cell frequencies were significantly higher for the IFN-γ monofunctional cells in HBV patients, but higher for TNF monofunctional CD4 T cells in HBV/HIV patients. While this difference was observed in the cytokine expression profile, the functionality analysis showed no significant difference. IFN-γ monofunctional HBV pol-reactive CD4 T cells were more frequent in HBV than HBV/HIV patients. The analysis of the functionality of HBV pol-reactive CD4 T cells revealed slightly, but not significantly less polyfunctional T cells in HBV than HBV/HIV patients. Since polyfunctionality is seen as a correlate of protection in general immunology especially regarding vaccination [522-524] and HBV immunology [462] and HIV immunology [525, 526], the data hints to HBV core-specific CD4 T cells of HBV patients as being more protective than in HBV/HIV co-infected patients, while it is the other way around to a limited degree in HBV pol-specific CD4 T cells. This could have an effect on the pathogenesis and disease progression of HBV/HIV co-infection.

Neither total HBV-reactive, nor HBV core-, env- and pol-reactive CD8 T-cell frequencies were significantly different in HBV and HBV/HIV patients. This implies that HIV infection does not induce changes in the magnitude of HBV CD8 T-cell immunity, although the T-cell responses are only determined in peripheral PBMC that might not translate directly to organ-specific e.g. liver-resident CD8 T cells [527-530]. Analyzing the magnitude of single cytokine (IFN-γ, IL-2, TNF)-secreting HBV core-, env- and pol-reactive CD8 T cells, the data

showed that HBV core-reactive CD8 T cells produced significantly more TNF in HBV than in HBV/HIV patients and that HBV pol-reactive CD8 T cells produced significantly more IFN-γ in HBV mono- than in HBV/HIV co-infection. This indicates a stronger antiviral function in HBV mono-infection. The polyfunctionality of HBV pol-reactive CD8 T cells was significantly different in HBV/HIV co- than HBV mono-infection. Taking the cytokine expression profile of HBV pol-reactive CD8 T cells into account, IFN-γ monofunctional cells were strongly and significantly lower in HBV/HIV patients, while TNF monofunctional CD8 T cells were slightly and significantly higher in HBV/HIV co- versus HBV mono-infection. Overall, monofunctionality of HBV pol-reactive CD8 T cells was higher in HBV than in HBV/HIV patients, implying a higher HBV protectivity in the HBV/HIV co-infection as polyfunctionality, but not monofunctionality is regarded as a correlate of protection.

Evaluating FEC-reactive T cells, frequencies of CD8 T cells were shown to be a lot higher than of CD4 T cells, which was reasonable since the FEC peptide pool is designed to challenge CD8 T-cell responses [519, 520]. Neither frequencies of CD4, nor CD8 FEC-reactive T cells were significantly different between HBV mono-, HBV/HIV co- and HIV mono-infected patients, which implies that FEC-reactive T cells seem not to be influenced by the HIV infection. HIV nef-reactive CD4 and CD8 T-cell frequencies were similar and not significantly different between HIV and HBV/HIV infected patients. Similarly to the results of the stimulation with the comparative antigen FEC, results upon HIV nef challenge were not different due to the HBV co-infection.

### 3.4 Correlations and immunopathogenesis

When correlating CD8 T-cell responses with CD4 counts, there was no clustering of neither HBV antigen-reactive, nor FEC and HIV nef reactive CD8 T-cell frequencies with high or low CD4 counts in this study. The CD4 count does therefore not seem to be a major dedicator of HBV-specific T-cell immunity. This might be different at an earlier point in the natural history of the HBV infection or even in the stage of AIDS (CD4 count < 200/µL), where CD4 T-cell help might be at critical tipping points for the sustainability of CD8 T-cell responses [531, 532].

This study reports ALT and AST serum liver enzyme levels to be significantly higher in HBV than in HBV/HIV patients. By contrast, liver disease progression and severity is reported to be higher in HBV/HIV co-infection [416]. HIV infection alone does not seem to have a correlation with increased liver enzyme levels [533]. Nevertheless, it was shown before that HBV co-infection does not lead to higher ALT, but only AST levels in comparison to HIV mono-infection [534], but that HBV/HIV co-infection is associated with higher liver toxicity [535]. The data indicate that the elevated serum liver enzyme levels in HBV patients are caused by an increased immunopathogenesis in comparison to HBV/HIV co-infection, although the antiviral therapy suppressed HBV DNA in both HBV mono- and HBV/HIV co-infected patients.

The frequency of peripheral HBV env-reactive CD8 T cells of HBV patients correlated negatively with rising ALT and AST levels. This might be caused by HBV env-specific CD8 T cells that migrated to the liver causing liver damage as described previously by Mala Maini et al. [203]. In HBV/HIV patients, frequencies of HBV env-reactive CD8 T cells were higher with rising ALT and AST levels hinting that liver damage might not be caused by HBV env-specific T cells due to dysfunction or decreased liver migration. HIV nef-reactive CD8 T cells of HBV/HIV patients did not correlate with rising ALT and AST levels showing that the frequencies of these T cells is not changed in different liver disease stages. By contrast, HIV patients had higher HIV nef-reactive CD8 T cells with rising ALT and significantly with rising AST levels. This could be due to a general T-cell activation upon a strong inflammatory environment or might also mirror the presence of liver-resident T cells although this was mainly caused by one patient's data and therefore needs more investigation. While HIV-specific T cells are not known to be causative for liver damage, liver inflammation in general is reported to be increased in HIV mono-infection and HBV/HIV co-infection in contrast to HBV/HIV naïve people [390].

### 3.5 Immune regulatory mechanisms and cells (Treg / T-cell exhaustion)

Regulatory T cells lead to dysregulation and suppression of immune responses during HBV infections, promoting disease progression and persistence [536]. In this study, median frequencies of $CD25^+$ $FoxP3^+$ regulatory CD4 T cells were higher (although not significantly) in HBV mono- than in HBV/HIV co-infected patients (Fig 38). Similarly, Sherman et al. showed no significant difference between Treg frequencies of HBV mono- and HBV/HIV co-infected patients, but that Treg were able to inhibit HBV-specific T-cell responses in patients with chronic hepatitis B independent of their HIV infection status [455]. It was already reported before that strong T-cell activation induces proliferation of regulatory T cells by negative feedback loops [537, 538] and shown that persistent HBV infection induces Treg [239, 539], which was confirmed by data from the present study as HBV mono-infected patients revealed significantly higher Tre frequencies than HBV/HIV naïve subjects, who had similar frequencies as HBV/HIV co-infected individuals. As HBV/HIV co-infection shows no quantitative induction of peripheral Treg as HBV mono-infection did in comparison to HBV/HIV naïve individuals, it is possible that an HIV infection-induced dampening of the immune response [540] might inhibit the HBV infection-linked proliferation of regulatory T cells. Although it was reported elsewhere that HIV does also infect regulatory T cells [541, 542], patients with HIV mono-infection (like with HBV/HIV co-infection) showed no differences in the frequency of regulatory T cells in comparison to HBV/HIV naïve individuals. Overall, Treg frequencies of HBV mono- and HBV/HIV co-infected patients were not significantly different, which could have been the cause for differences in the cytokine expression profile of patients with HBV mono- or HBV/HIV co-infection.

Aside from immune cell populations that are able to inhibit T-cell activation, proliferation, differentiation or T-cell responses, there are regulatory mechanisms linked to T-cell dysfunction. Receptors of inhibitory molecules on CD8 T cells are often associated with T-cell dysfunction and T-cell exhaustion. Various surface markers of T-cell exhaustion were thereby identified previously. CD39 (with CD73) was shown to catabolize ATP to adenosine, which inhibits T-cell responses [543]. Aside from Treg in HBV [82] and HIV infection [544], CD39 is expressed by antigen-experienced CD8 T cells in tumors and virus infections like HIV [81, 545-548]. TIGIT was found to block T-cell activation and to be upregulated in T-cell exhaustion [78]. General expression of PD-1 is reported for activated T cells, while T cells with high levels of $PD\text{-}1^{high}$ expression are shown to be dysfunctional, exhausted T cells

[549-551]. Frequencies of the receptors CD39, TIGIT and PD-1$^{high}$ were detected on antigen-experienced CD8 T cells in this study to ascertain the presence of dysfunctional and functional T cells in HBV mono- and HBV/HIV co-infection, which are linked to HBsAg seroconversion (referred to as functional cure) [160, 204].

The surface expression of the exhaustion marker CD39 on antigen-experienced CD8 T cells was (non-significantly) higher in HBV/HIV co- than HBV mono-infection. While CD39$^{+}$ CD8 cells were reported elsewhere as liver-resident memory T cells in HBV-infected patients, they were not found in the blood of the respective patients [552]. In a comparison of patients with active and inactive chronic hepatitis B, no differences in CD39 expression on CD8 T cells or T-cell exhaustion-associated CD8 T cells were found [553]. In line with CD39 being published as an exhaustion marker on T cells in HIV infected patients [81], CD39 expression on antigen-experienced CD8 T cells of HBV/HIV co-infected patients in this study was significantly higher than of HBV/HIV naïve subjects. By contrast, levels of HIV mono-infected patients were similar to HBV/HIV naïve individuals and also significantly lower than of HBV/HIV co-infected patients. TIGIT levels, although slightly higher in HBV/HIV patients, were not significantly different to HBV mono-infected patients in this study. Frequencies of TIGIT$^{+}$ antigen-experienced CD8 T cells of HBV/HIV naïve subjects were similar to HBV mono- and HBV/HIV co-infected patients. TIGIT$^{+}$ CD8 T cells were associated with the pathogenesis and progression of patients with HBV infection-related HCC [554], as well as HIV/AIDS [555]. No previous data on TIGIT frequencies in HBV/HIV co-infections was available. Whereas there were no significant differences of PD-1$^{high}$ frequencies between all cohorts in this study, only a subgroup of HBV/HIV co-infected patients had high levels of PD-1$^{high}$ expression. Others reported previously that PD-1 is upregulated on total and HBV-specific CD8 T cells of patients with chronic hepatitis B [556], while no information for HBV/HIV co-infection was available [390]. It was shown before that serum PD-1 levels in HBV mono-infected patients were a lot (but not significantly) higher than in patients with HBV/HIV co-infection, which were similar to HBV/HIV naïve individuals [505]. Additionally, PD-1 augmentation was shown to induce proliferation of HBV-specific T cells in HBV/HIV co-infection [455].

SPADE tree analysis [557, 558] showed a co-clustering of the expression of transcription factors T-bet and Eomes with distinct PD-1$^{+/-}$ and CD127$^{+/-}$ subpopulations. The expression pattern of CD127 and PD-1 can be used to define the status of different T-cell

functions [559]. The frequencies of CD8 T-cell populations with defined T-bet and Eomes expression were therefore compared in HBV mono- and HBV/HIV co-infection. Overall, T-bet$^{high}$ expressing antigen-experienced CD8 T cells were significantly more frequent in HBV mono- than in HBV/HIV co-infected patients in this study. Frequencies of T-bet$^{high}$ CD8 T cells of HBV/HIV co-infected patients were divided in two groups. The group with higher frequencies was comparable to HBV mono-infected patients, while the lower was similar to HIV mono-infected patients. Reports for chronic LCMV showed Eomes deficiency to be linked to an active and antiviral CD8 T-cell phenotype [560]. HIV/AIDS elite controllers reveal high T-bet levels [561], while the loss of the transcription factor is observed in patients with chronic HIV infection [562]. T-bet levels of T cells in patients with acute hepatitis B were reported to be high, while they were lost in chronic hepatitis B [563]. The subpopulation of exhausted CD8 T cells with Eomes$^{+}$ T-bet$^{low}$ expression was comparable in HBV mono- and HBV/HIV co-infected patients in this study. Only a subgroup of very few HBV/HIV co-infected patients had higher levels of Eomes$^{+}$ T-bet$^{low}$ CD8 T cells similar to HIV mono-infected patients. The Eomes$^{+}$ T-bet$^{low}$ phenotype of T cells is associated with T-cell exhaustion as T-bet deficiency was found in exhausted T cells in chronic LCMV infection [564]. High levels of Eomes and low expression of T-bet was shown to be linked to the loss of polyfunctional CD8 T cells in HIV infection [565] and progressive HIV/AIDS [561]. Exhausted HBV polymerase-specific CD8 T cells showed low T-bet, but high Eomes expression in chronic hepatitis B [237]. Summarizing this data, a functional phenotype was found in HBV mono-infection on the transcriptional level.

Multi-dimensional visualization in this study was generated by the tSNE algorithm [566] showing T-cell clusters with similar T-cell exhaustion and memory phenotype and revealed distinct populations of PD-1$^{high}$, CD127$^{-}$ Tcf-1$^{-}$ terminally exhausted and PD-1$^{+}$ CD127$^{+}$ Tcf-1$^{+}$ progenitor exhausted T cells, which were defined before [60, 62]. The population of PD-1$^{+}$ CD127$^{+}$ Tcf-1$^{+}$ progenitor exhausted T cells was comparable in HBV mono- and HBV/HIV co-infected patients, whereas the PD-1$^{high}$, CD127$^{-}$ Tcf-1$^{-}$ terminally exhausted CD8 T-cell population was a lot more prevalent in HBV/HIV co-infected individuals. While T-cell exhaustion was already reported for HBV infection [567, 568] and HBV/HIV co-infection [505], it was found that exhausted T cells in patients with chronic hepatitis B had the memory-like, precursor phenotype with a limited remaining function and proliferative capacity, but not the terminally exhausted CD8 T cells [237]. Studies comparing the phenotype and exhaustion status of CD8 T cells of different HBV antigen-specificity

revealed HBV pol-specific, but not HBV core-specific CD8 T cells to be terminally exhausted implying that core-specific T cells could be beneficiaries of functional restoration [569].

The here reported results show no significant impact of HIV co-infection on the population of regulatory CD4 T cells and the expression of exhaustion marker levels on CD8 T cells. Nevertheless, the data show terminally exhausted CD8 T-cell phenotype in HBV/HIV co-infection in comparison to HBV mono-infection, which suggests that conventional immunotherapy might not be effective in patients with HBV/HIV co-infection. By contrast, T-cell exhaustion should be considered for the development and application of novel immunotherapy approaches.

### 3.6 Limitations of the study

However, the present study comprises multiple limitations that should be discussed below.

A major limitation of this study could be the patient numbers which are overall high enough in each cohort to support statistically relevant analysis, whereas subsets of antigen-reactive responding PBMC are limited after application of the threshold. An increase in sample size could strengthen statistical analysis for all subsets if more responders were to be included.

The provenance could be an indication for the HBV genotypes patients are infected with. While in Europe and Africa genotypes A, D and E are most prevalent, patients from Asia are also likely to carry genotypes B or C [146]. In this study, HBV-derived overlapping peptide pools were used that are based on HBV genotype D. Granting the genotype of the patients was not tested in the clinics, sequence analysis revealed more than 85 – 95 % sequence homology between the HBV proteins of genotypes D and B. Genotypes B and C are very similar, which is also true for genotypes A and D. An analysis of the sequences for genotype E showed 84 – 91 % homology with genotype B, with which it showed the lowest match/conformity. Epitope mapping to the genotype sequence comparison revealed only minimal differences in the epitopes, while important anchor positions were only different in one epitope of both the HBV env and the HBV core antigens (Supplemental tables 2 – 11).

Although timepoints of the patient's hepatitis B and HIV/AIDS diagnosis are known, the sequence of contracting HBV and HIV is unknown for the HBV/HIV co-infected patients. This might be very different in subjects with different origins. While HBV contraction occurs in Africa very often during child birth, HIV is transmitted with the first sexual contact during early adolescence [330]. In the Western World in contrast both HIV and HBV are mainly contracted during adulthood [1, 330]. The sequence of infections could majorly impact the natural history of the diseases with HBV-infection permitted immune activation rendering more CD4 T cells susceptible to productive HIV infection and boosting HIV replication, while HIV infection affects T-cell responses that are central to the control and resolution of HBV infections [208, 390, 570].

Isolation of PBMC from HBV/HIV co-infected patients was performed in Munich after shipping sodium citrate CPT tubes overnight, while PBMC from HBV mono- and HIV mono-infected patients were directly isolated from EDTA whole blood with pancoll separation at the respective study side. Several studies showed that CPT- and pancoll-based

separations are comparable in regards to the PBMC recovery and following T-cell function capacity leaving only the transport of the samples as a limitation in this study [571-573].

Differences in HBV-reactive T-cell responses from HBV/HIV co-infected and HBV mono-infected patients after PBMC rechallenge with overlapping peptide pools derived from HBV core, envelope and polymerase were analyzed. HBx-reactive T-cell responses were not determined in this study and are therefore not included in the total HBV-specific T-cell response.

With the staining of IFN-γ, IL-2 and TNF produced by CD4 and CD8 T cells, only $T_h1$ T-cell responses were detected by flow cytometry-based ICS. No effect of the HIV co-infection could therefore be determined on HBV-reactive anti-inflammatory responses.

### 3.7 Outlook

The present analysis of CD4 and CD8 T-cell responses upon stimulation with HBV protein-derived peptide pools was comprehensive for the determination of differences in HBV mono- and HBV/HIV co-infected patients. Going further, HBV X-reactive CD4 and CD8 T cells could be analyzed in a similar way as HBV core-, envelope- and polymerase-reactive T cells by flow cytometry-based intracellular cytokine staining to gather this information about all HBV antigen.

While antigen-specific T cells were detected by the production of the cytokines IFN-γ, IL-2 and TNF, the cytotoxicity can rather be analyzed by quantification of molecules such as perforin or granzyme B [9]. The effective killing capacity of T cells of different phenotype and specificity could be determined with co-culture experiments e.g. with an incucyte [574, 575] or xCelligence system [576].

If the HIV-specific T cells should further be analyzed, reactivity upon stimulation with peptide pools derived from the other HIV proteins like HIV Env, Pol, Vpu, Vpr, Vif could be determined.

To analyze cross-reactive polymerase-specific T cells, the cytokine production of T cells could be analyzed in parallel with an HIV polymerase- and an HBV polymerase-derived peptide pool. Sorting these T cells with HIV pol- or HBV pol-specific tetramers could allow us to test the sorted cells for cross-reactivity upon stimulation with the other peptide pool either in flow cytometry or Fluorospot analysis.

While the frequency of peripheral regulatory T cells was determined, the functionality of Treg was not determined. It would therefore be interesting to analyze the cytokine production (e.g. IL-10, TGF-β) or even the transcriptome of Treg and compare this for HBV mono- and HBV/HIV co-infection.

T-cell exhaustion and transcription factors of HBV-specific T cells could be analyzed by sorting the cells with HBV core-, env- and pol-tetramers and analyzing them with flow cytometry or RNAseq, but would be limited by the low precursor frequency of those cells.

Single-cell sorting and RNAseq could be applied to determine the transcriptome of T cells with different antigen-specificity and detect which cells are infected with HIV. T-cell

transcriptomics does therefore not only allow the analysis of changes in the different cohorts, but also distinguish which effect is caused only in HIV-infected cells and which are bulk effects on the whole T-cell population.

While differences in the presence of T cells with phenotypes that are typical for exhausted T cells were found, it remains elusive if changes in the cytokine expression profile of HBV-reactive T cells are mainly caused by exhaustion or by missing priming of the specific T cells in HBV/HIV co-infection. This could be caused by HIV infection-induced downregulation of HLA, TCR and CD4 [496, 577-580]. Due to the fact that HIV cannot only infect CD4 T cells, but also macrophages and dendritic cells [581], an inhibition of the antigen presentation and thereby lowered priming and activation of CD4 and CD8 T cells are possible. Analysis of antigen-presentation is quite complicated, but possible with mass spectrometry.

Liver needle aspiration would be valuable to determine the HBV-specific T-cell immunity in the liver that could be complimentary to the information about peripheral T cells. This is also true for populations of some antigen-specific T cells that do not get lost by apoptosis, but migrate from the periphery to the liver [582]. This precious material should rather be analyzed with RNAseq instead of flow cytometry to determine the transcriptomics of this immunological liver environment.

A longitudinal study would be informative regarding the development of T-cell responses, reveal the possible loss of the expression of certain antigens and rising levels of inhibitory signals and markers. Most precious were longitudinal samples from a patient with either HBV mono- or HIV mono-infection who contracts the other infection and therefore becomes HBV/HIV co-infected during the study.

To enlighten the mechanisms and backgrounds behind spontaneous clearance of HBV infections, it is not only worthwhile to compare HBV-specific T cells of patients with spontaneously cleared hepatitis B to individuals with the chronic disease, but also take HIV co-infection into the picture. Since it's observed in the clinic that HBV/HIV co-infected patients have higher rates of spontaneous clearance [429], it would be worthwhile to compare robust cohorts of patients with HIV infection and HBsAg seroconversion to

patients with active HBV/HIV co-infection to persistent HBV mono-infection and spontaneously cleared HBV mono-infection.

### 3.8 Conclusion

This study reports that HBV/HIV co-infection goes along with significant changes in the HBV-specific T-cell cytokine expression profile of HBV specific T cells. Frequencies of Treg and peripheral HBV-reactive CD4 and CD8 T cells were not affected, whereas response rates to the HBV protein-derived peptide pools as well as total CD8 T cells producing IFN-γ, IL-2 and TNF and the cytokine expression profile of CD4 and CD8 T cells were significantly different. Exhausted CD8 T cells in HBV/HIV co-infected patients had a terminally exhausted phenotype, while HBV mono-infected patients revealed a memory-like exhausted T-cell phenotype.

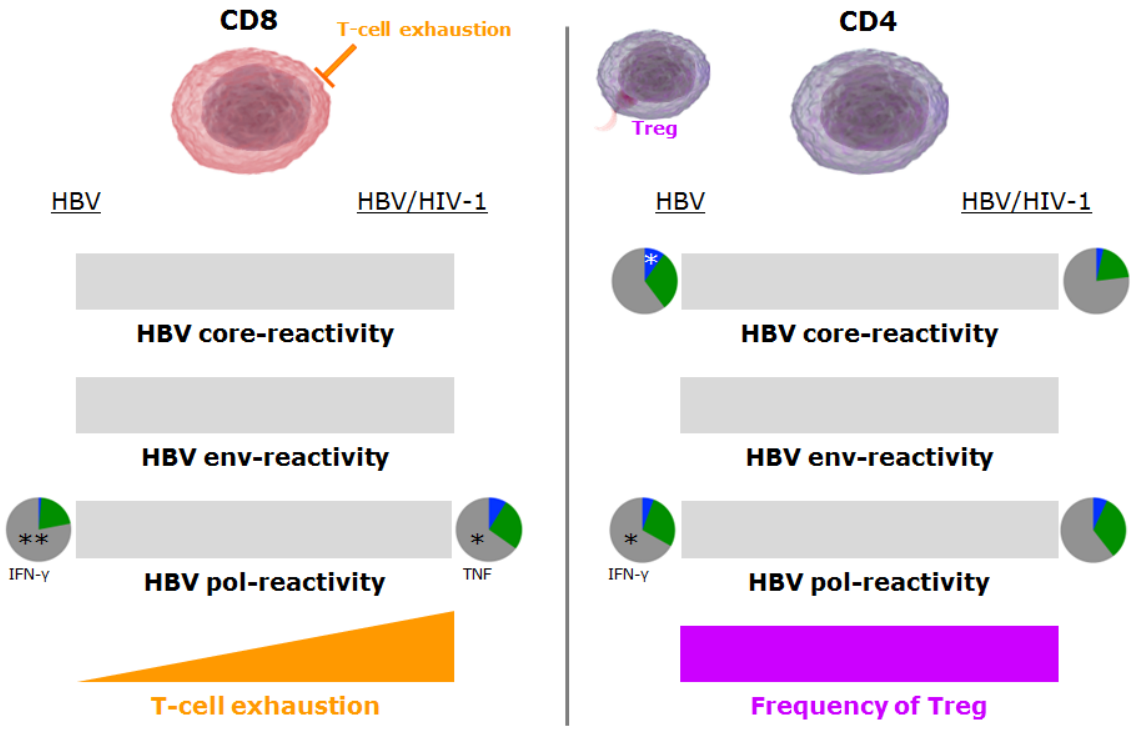


Figure 43: **Graphical conclusion**. HBV-specific CD4 and CD8 T-cell frequencies were not significantly different between HBV mono- and HBV/HIV co-infected patients. Nevertheless, the study reports significant differences in the cytokine expression profile of HBV-reactive CD4 and CD8 T cells between both cohorts. While no difference in the frequency of Tregs was found, HBV mono-infected patients had a more functional phenotype regarding the expression of transcription factors and the memory-like phenotype of the exhausted T cells. By contrast, HBV/HIV co-infected patients showed a higher prevalence of terminally exhausted T cells.

This study therefore furthers the understanding of HBV/HIV co-infection and challenges new hypotheses that HIV co-infection does not deplete HBV-specific T cells, but modulates their cytokine release e.g. through the induction of a terminally exhausted phenotype. Following these and underlining the here reported results with new technologies like RNA-seq and fine liver needle aspiration to unveil pathogenic mechanisms in the patients, can give insight into immunopathogenesis of HBV mono- and HBV/HIV co-infection alike, but also reveal key factors of spontaneous HBV clearance. This could enable the development of future therapies to fight the health threat that is chronic hepatitis B.

## 4. Abbreviations

Table 3: **Abbreviations used in this work**

| | |
|---|---|
| 'a' determinant | Neutralizing epitope in the HBV surface antigen |
| (L) HBsAg | large HBV surface protein |
| (M) HBsAg | Medium HBV surface protein |
| (S) HBsAg | Small HBV surface antigen |
| * | $p \leq 0.05$ |
| ** | $p \leq 0.01$ |
| *** | $p \leq 0.001$ |
| °C | degree Celsius |
| × g | times gravity |
| 3' end | Three prime end; the tail end of the DNA |
| AA | amino acid |
| AASLD | American Association for the Study of Liver Diseases |
| adr | One of the HBV serotypes |
| adw | One of the HBV serotypes |
| AIDS | Acquired immunodeficiency syndrome |
| AlexaFluor647 | Alexa Fluor dye emitting light at 647 nm |
| AlexaFluor700 | Alexa Fluor dye emitting light at 700 nm |
| ALT (GPT) | alanine aminotransferase |
| ANOVA | Analysis of variance (Kruskall-Wallis test) |
| anti-HBc | HBcAg-specific antibodies |
| anti-HBe | HBeAg-specific antibodies |
| anti-HBs | HBsAg-specific antibodies |
| APC | antigen presenting cell |
| APOBEC | apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like |
| ART | antiretroviral therapy |
| AST (GOT) | aspartate aminotransferase |
| ATP | Adenosine triphosphate |
| ayr | One of the HBV serotypes |
| ayw | One of the HBV serotypes |
| AZT | Zidovudine |
| B-cell | Bursa cells |
| BAF | BRG1- or BRM-associated factors (SWItch/Sucrose Non-Fermentable) |
| BB515 | Brilliant Blue dye emitting light at 515 nm |
| BFA | Brefeldin A |
| Bim | BCL-2 interacting mediator of cell death |
| bp | Base pairs |

| | |
|---|---|
| BUV395 | Brilliant Ultra-Violet dye emitting light at 395 nm |
| BV421 | Brilliant Violet dye emitting light at 421 nm |
| BV510 | Brilliant Violet dye emitting light at 510 nm |
| BV605 | Brilliant Violet dye emitting light at 605 nm |
| BV711 | Brilliant Violet dye emitting light at 711 nm |
| BV786 | Brilliant Violet dye emitting light at 786 nm |
| C terminus | Carboxyl end of a protein |
| cART | combination-antiretroviral therapy |
| cccDNA | covalently closed circular DNA |
| CCR5 | C-C chemokine receptor type 5 |
| CCR7 | C-C chemokine receptor type 7 |
| CD | cluster of differentiation |
| CD3 cells | T cells |
| CD4 T cells | Mostly T helper cells, but also regulatory T cells |
| CD40L | CD40 ligand |
| CD8 T cells | Mostly cytotoxic T cells |
| CDC | Center for disease control and prevention of the USA |
| Cfp1 | CxxC finger protein 1 |
| CHB | chronic hepatitis B |
| CMV | cytomegalovirus |
| CpAM | core protein allosteric modulators |
| CpG | cytosine – phosphate – guanine |
| CPT | Cell preparation tube |
| crispr/cas9 | clustered regularly interspaced short palindromic repeats/ CRISPR associated protein 9 |
| CTL | cytotoxic T lymphocyte |
| CTLA-4 | cytotoxic T-lymphocyte-associated protein 4 |
| CXCR3 | Cysteine – X – Cysteine chemokine receptor 3 |
| CXCR4 | Cysteine – X – Cysteine chemokine receptor 4 |
| DAA | Direct acting antivirals |
| DAMPs | damage-associated molecular patterns |
| DC | dendritic cell |
| DC-SIGN | Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin |
| DGVS | Deutsche Gesellschaft für Gastroenterologie, Verdauungs- und Stoffwechselkrankheiten; German society for gastroenterology, digestion and metabolism diseases |
| DMSO | Dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |
| dsDNA | Double-stranded DNA |
| EBV | Epstein–Barr virus |
| EDTA | Ethylenediaminetetraacetic acid |

| | |
|---|---|
| eFluor450 | eFluor dye emitting light at 647 nm |
| ELISA | enzyme-linked immunosorbent assay |
| ELISPOT | enzyme-linked immune absorbent spot |
| EMA | European medicines agency |
| ENH1 | HBV transcription enhancer region 1 |
| ENH2 | HBV transcription enhancer region 2 |
| Eomes | Eomesodermin |
| ER | endoplasmic reticulum |
| EU | European Union |
| FACS | Fluorescence-activated cell sorting |
| Fas ligand | first apoptosis signal ligand |
| Fc | fragment crystallizable |
| FCS | fetal calf serum |
| FDA | US food and drug administration |
| FEC | Flu-, EBV- and CMV-derived peptide pool |
| FITC | Fluorescein isothiocyanate |
| Flu | Influenza virus |
| FoxO1 | Forkhead box protein O1 |
| FoxP3 | Forkhead box P3 |
| gag | Group-specific antigen (protein of e.g. HIV) |
| GI tract | Gastrointestinal tract |
| gp120 | envelope protein |
| gp41 | envelope protein |
| grzB | granzyme B |
| h | hours |
| HAART | highly active antiretroviral therapy |
| HBc | HBV core protein |
| HBeAg | hepatitis B virus e antigen |
| HBsAg | hepatitis B virus surface antigen |
| HBSP | hepatitis B spliced protein |
| HBV | hepatitis B virus |
| HBV env | HBV envelope protein |
| HBV/HIV | Coinfection with HBV and HIV (Cohort) |
| HBx | hepatitis B virus x protein |
| HC | healthy control; HBV/HIV naïve |
| HCC | hepatocellular carcinoma |
| HCV | Hepatitis C virus |
| HDACi | Histone-deacetylase inhibitors |
| HIV | HIV infected patients (Cohort) |
| HIV-1 | Human immunodeficiency virus type 1 |

| | |
|---|---|
| HIV-2 | Human immunodeficiency virus type 2 |
| HIV/AIDS | Disease name of the HIV infection, which is eventually progressing to Acquired immunodeficiency syndrome |
| HIV/rHBV | HIV infection and resolved HBV (Cohort) |
| HLA | Human leukocyte antigen |
| HMT | Histone methyltransferase |
| HNF-1α | hepatocyte nuclear factor 1 homeobox A |
| HNF-4 | Hepatocyte Nuclear Factor 4 |
| HTLV-1 | human T-lymphotropic Virus 1 |
| ICE-HBV | International Coalition to Eliminate HBV |
| ICS | intracellular cytokine staining |
| IFITM | Interferon induced transmembrane proteins |
| IFN-α | Interferon alpha |
| IFN-β | Interferon beta |
| IFN-γ | Interferon gamma |
| IgM | Immunoglobulin macro |
| IL-10 | Interleukin 10 |
| IL-12 | Interleukin 12 |
| IL-15 | Interleukin 15 |
| IL-2 | Interleukin 2 |
| IL-32 | Interleukin 32 |
| IL-4 | Interleukin 4 |
| IL-6 | Interleukin 6 |
| IL-7 | Interleukin 7 |
| IR | infrared |
| IRD | immune reconstitution disease |
| ISG | Interferon stimulated gene |
| ISG15 | Interferon stimulated gene 15 |
| ISG20 | Interferon stimulated gene 20 |
| IU | International Units |
| JAK/STAT | Janus kinases/ signal transducer and activator of transcription proteins |
| kB | kilo base |
| LCMV | Lymphocytic Choriomeningitis virus |
| LPS | Lipopolysaccharides |
| LRAs | Latency reversing agents |
| LSEC | liver sinusoidal endothelial cells |
| LTR | long-terminal repeat |
| Mann-Whitney U test | Also known as Wilcoxon rank-sum test |
| MAVS | Mitochondrial antiviral-signaling protein |
| Mda5 | melanoma differentiation-associated protein 5 |

| | |
|---|---|
| MHC (MHCI, MHCII) | major histone compatibility complex |
| MIATA | minimal information about T-cell assays |
| min | Minutes |
| mL | milliliter |
| MRI | Klinikum Rechts der Isar in Munich |
| mRNA | Messenger RNA |
| Mx1 | Myxovirus resistance protein |
| n = | Natural number/a count |
| N terminus | Amine beginning of a protein |
| nef | Negative Regulatory Factor e.g. of HIV |
| NF-κB | nuclear factor kappa-light-chain-enhancer of activated B cells |
| NIR | LIVE/DEAD Near-IR staining |
| NK cells | Natural killer cells |
| nm | nanometer |
| NNRTI | Non-nucleoside reverse-transcriptase inhibitors |
| NRF1 | nuclear respiratory factor 1 |
| NRTI | Nucleoside/nucleotide analog reverse-transcriptase inhibitors |
| ns | Non-significant |
| nt | nucleotides |
| NTCP | sodium taurocholate co-transporting polypeptide |
| OAS1 | 2'-5'-oligoadenylate synthetase 1 |
| Oct-1 | Octamer transcription factor 1 |
| ORF | open reading frame |
| *p* | probability |
| p.i. | Post infection |
| p160 | Protein with 160 kDa (gag-pol polyprotein of HIV) |
| p17 | Protein with 17 kDa (matrix protein of HIV) |
| p24 | Protein with 24 kDa (capsid of HIV) |
| p6 | Protein with 6 kDa (protein in Pol of HIV) |
| PAMPs | pathogen-associated molecular patterns |
| PBMC | peripheral blood mononuclear cell |
| PBS | Phosphate buffered saline |
| pcRNA | precore mRNA |
| PD-1 | programmed cell death protein 1 |
| PD-L1 | PD-ligand 1 |
| PE | Phycoerythrin |
| PE-CF594 | Phycoerythrin emitting light at 594 nm |
| PE-Cy7 | Phycoerythrin coupled to Cyanine 7 |
| Peg-IFN-α | pegylated interferon-α |
| PenStrep | Penicillin-Streptomycin |

| | |
|---|---|
| PEP | post-exposure prophylaxis |
| PerCP | Peridinin-Chlorophyll-Protein |
| PerCP-e710 | Peridinin-Chlorophyll-Protein emitting light at 710 nm |
| pgRNA | pregenomic RNA |
| PI | Protease inhibitor |
| PIC | pre-integration complex |
| PML | promyelocytic leukemia protein |
| pol | Polymerase (of HBV or HIV) |
| polyA | Poly Adenosine |
| preCore | First ORF in the Core transcript of HBV |
| PrEP | Pre-Exposure Prophylaxis |
| preS1 | First ORF in the Surface transcript of HBV |
| preS2 | Second ORF in the Surface transcript of HBV |
| PRRs | Pathogen recognition receptors |
| *r2* | Correlation coefficient |
| R5 | M-tropic |
| rcDNA | relaxed circular DNA |
| rev | regulator of expression of virion proteins |
| RIG-I | retinoic acid-inducible gene I |
| RKI | Robert Koch Institute |
| RNA | ribonucleic acid |
| RNaseH | Ribonuclease H |
| RNAseq | RNA sequencing (transcriptomics) |
| RPMI1640 | Roswell Park Memorial Institute medium |
| RRE | Rev-responsive element |
| RT | Reverse transcriptase |
| RXR-alpha | Retinoid X receptor alpha |
| S-CAR | Chimeric antigen receptor specific for the HBV envelope protein |
| SAMHD1 | SAM domain and HD domain-containing protein 1 |
| siRNA | Small interfering RNA |
| SIV | simian immunodeficiency virus |
| SLIP element | Frameshift element in HIV |
| SOP | standard operating procedure |
| SPADE | spanning-tree progression analysis for density-normalized events |
| SPICE | Simplified Presentation of Incredibly Complex Evaluations |
| ssRNA | Single-stranded RNA |
| STAT1 | Signal transducer and activator of transcription 1 |
| STIKO | Ständige Impfkommission |
| SUMOylation | Small Ubiquitin-like Modified |
| T cell/lymphocytes | Thymus cell |

| | |
|---|---|
| T-bet | T-box expressed in T cells |
| t-HBV | treated HBV infected patients (Cohort) |
| T-tropic | HIV virions that use the coreceptor CXCR4 |
| TAR | trans-activation response |
| tat | Transactivator of transcription (HIV protein) |
| Tcf-1 | T cell factor 1 |
| TCM | central memory T cells |
| TCR | T-cell receptor |
| TEM | effector memory T cells |
| TEMRA | terminally differentiated effector memory T cells |
| Tfh | follicular helper T cell |
| TGF-β1 | transforming growth factor beta 1 |
| $T_h$ ($T_h1$, $T_h2$) | T helper cell |
| TIGIT | T cell immunoreceptor with Ig and ITIM domains |
| TLR | Toll-like receptors |
| tn-HBV | treatment-naïve HBV infected patients (cohort) |
| TNF (TNF-α) | tumor necrosis factor |
| Tox | Thymocyte selection-associated high mobility group box protein |
| Treg | Regulatory T cells |
| Trim5a | Tripartite motif-containing protein 5 |
| TRM | tissue-resident memory T cells |
| tRNA | Transfer RNA |
| TSCM | memory stem T cells |
| tSNE | t-distributed stochastic neighbour embedding |
| US | United States of America |
| USA | United States of America |
| vif | Viral infectivity factor |
| vpr | Viral Protein R |
| vpu | Viral Protein U |
| w/o | without |
| WHO | world health organization |
| X | Hepatitis B virus X protein |
| X4 | HIV virions that use the coreceptor CXCR4 |
| YFV | yellow fever virus |
| µg | microgram |
| µL | microliter |
| µm | micrometer |

## 5. Material

Table 4: **Machines, Equipment, Chemicals & Software**

| Material Specification | Producer, place of Business |
|---|---|
| **Machines & Equipment** | |
| Plate centrifuge Rotanda 460R | Hettich, Tuttlingen, Germany |
| Table centrifuge Mikro 200R | Hettich, Tuttlingen, Germany |
| BD LSRFortessa™ cell analyzer | BD, Franklin Lakes, USA |
| ViCell XR cell counter | Beckman Coulter, Krefeld, Germany |
| Bead bath WNB14 | Memmert, Schwabach, Germany |
| Mr. Frosty freezing devices | Thermo Fisher Scientific, Waltham, USA |
| Eppendorf Research® plus Liquid Handling Pipette (10 µL, 20 µL, 200 µL, 1 mL) | Eppendorf, Hamburg, Germany |
| Pipette controllers accu-jet pro | Brand, Wertheim, Germany |
| **Plastics** | |
| Cellstar tubes (50 mL, 15 mL) | Greiner Bio-One, Frickenhausen, Germany |
| Cellstar Serological Pipette (10 mL) | Greiner Bio-One, Frickenhausen, Germany |
| Clearline filter pipette tips (10 µL, 20 µL, 200 µL, 1 mL) | Biosigma, Valetta, Italy |
| Fisherbrand SureOne pipette tips | Fisher Scientific, Schwerte, Germany |
| Lithium heparin S-Monovette tubes | Sarstedt, Nümbrecht, Germany |
| Safety-Lok & Vacutainer | BD, Franklin Lakes, USA |
| Sodium citrate CPT tubes | BD, Franklin Lakes, USA |
| Nunc™ Cryovials | Thermo Fisher Scientific, Waltham, USA |
| Falcon™ cell strainers | Corning Inc, Corning, USA |
| Titertube micro test tubes | Bio-Rad, Hercules, California, USA |
| Falcon round bottom polystyrene test tube | Corning, Corning, New York, USA |
| Falcon U-bottom 96-well plates | Fisher Scientific, Hampton, USA |
| **Chemicals** | |
| PBS (Phosphate buffered saline) | Life Technologies, Darmstadt, Germany |
| Human Pancoll | PAN-BIOTECH, Aidenbach, Germany |

| | |
|---|---|
| Dimethyl sulfoxide (DMSO) | Sigma-Aldrich, Steinheim, Germany |
| Roswell Park Memorial Institute medium (RPMI1640) with GlutaMAX | Life Technologies, Darmstadt, Germany |
| Fetal calf serum (FCS) | Life Technologies, Darmstadt, Germany |
| Penicillin-Streptomycin (PenStrep) | Life Technologies, Darmstadt, Germany |
| Brefeldin A (BFA) | Sigma-Aldrich, St. Louis, USA |
| Parafilm | Bemis, Neenah, US |
| eBioscience™ Flow Cytometry Staining buffer | Life Technologies, Darmstadt, Germany |
| eBioscience™ Intracellular Fixation buffer | Life Technologies, Darmstadt, Germany |
| eBioscience™ FoxP3/Transcription factor Fixation buffer | Life Technologies, Darmstadt, Germany |
| eBioscience Permeabilization buffer | Life Technologies, Darmstadt, Germany |
| UltraComp eBeads™ Compensation Beads | Life Technologies, Darmstadt, Germany |
| ArC™ Amine Reactive Compensation Beads | Life Technologies, Darmstadt, Germany |
| Cytometer, Setup & Tracking beads | BD, Franklin Lakes, USA |

### Peptide pools

| | |
|---|---|
| HBV core protein-derived | Genaxxon, Ulm, Germany |
| HBV polymerase protein-derived | Genaxxon, Ulm, Germany |
| HBV surface protein-derived | Genaxxon, Ulm, Germany |
| HIV-nef ultra peptide pool | JPT peptide technologies, Berlin, Germany |
| FEC peptide pool | NIBSC, Potters Bar, UK |

### Software

| | |
|---|---|
| FlowJo 10 | FlowJo, LLC, Ashland, Orgeon, USA |
| GraphPad Prism 7 | GraphPad Software, San Diego, USA |
| SPICE 6 | niaid.github.io/spice |
| RStudio 1.1.463 | RStudio, Boston, USA |
| Microsoft Office | Microsoft Corporation, Redmond, USA |
| Adobe Illustrator | Adobe Inc., San Jose, California, USA |
| Adobe InDesign | Adobe Inc., San Jose, California, USA |

Table 5: **Antibodies and cell staining**

| Antibodies | Clone | Dilution | Producer |
|---|---|---|---|
| LIVE/DEAD™ Fixable Near-IR Dead Cell Stain | | 1:200 | Life Technologies |
| LIVE/DEAD™ Fixable Blue Dead Cell Stain | | 1:100 | Life Technologies |
| CCR7 BB515 | 3D12 | 1:20 | BD |
| CD127 BV605 | A019D5 | 1:50 | Biolegend |
| CD127 PE | hIL-7R-M21 | 1:80 | BD |
| CD25 BV786 | MA251 | 1:50 | BD |
| CD28 Pure | L293 | 1 µg/mL | BD |
| CD3 BUV395 | SK7 | 1:50 | BD |
| CD3 BV510 | SK7 | 1:40 | Biolegend |
| CD3 FITC | SK7 | 1:50 | BD |
| CD39 BV711 | TU66 | 1:67 | BD |
| CD4 APC | OKT4 | 1:25 | eBioscience |
| CD4 PE-CF594 | RPA-T4 | 1:20 | eBioscience |
| CD4 PerCP | M-T466 | 1:25 | Miltenyi Biotec |
| CD45RA BV510 | HI100 | 1:100 | Biolegend |
| CD8 AlexaFluor700 | HIT8a | 1:100 | Biolegend |
| CD8 PE-CF594 | RPA-T8 | 1:50 | BD |
| CD8 PE-Cy7 | RPA-T8 | 1:200 | eBioscience |
| Eomes PerCP-e710 | WD1928 | 1:20 | eBioscience |
| FoxP3 eFluor450 | 236A/E7 | 1:33 | eBioscience |
| IFN-γ AlexaFluor700 | B27 | 1:1600 | BD |
| IL-2 FITC | MQ1-17H12 | 1:32 | eBioscience |
| PD-1 BV421 | EH12.2H7 | 1:20 | Biolegend |
| T-bet PE | eBio4B10 | 1:33 | eBioscience |
| Tcf-1 AlexaFluor647 | 7F11A10 | 1:20 | Biolegend |
| TIGIT PE-Cy7 | MBSA43 | 1:100 | eBioscience |
| TNF eFluor450 | Mab11 | 1:20 | eBioscience |

## 6. Methods

The authors of this study followed the MIATA (minimal information about T-cell assays) framework [583-585]. Therefore, information on study samples and handling thereof is given as proposed by the MIATA guidelines (http://miataproject.org/) [585].

All experimental steps were performed according to our established standard operating procedures (SOP) referring to the isolation of peripheral blood mononuclear cells (PBMC), cell counting, cryoconservation of PBMC, resting and the staining and flow cytometric analysis for the detection of T-cell responses, T-cell phenotypes, T-cell exhaustion and regulatory T cells.

### 6.1 Patient cohorts

Patients with chronic hepatitis B, patients with HIV-1 and patients with HIV-1 as well as persistent or resolved HBV infection were recruited at the Klinikum Rechts der Isar in Munich (MRI), the Universitätsklinikum Freiburg and the Medizinische Klinik der Universität Bonn. Cohort background data and clinical information were collected in the department of internal medicine II, University Hospital rechts der Isar and the department of medicine I, University Hospital Bonn and the department of Medicine II, Medical Center - University of Freiburg and are summarized in table 2. Patients were excluded when they were therapy-naïve and/or showed HBV DNA loads above 2000 IU/mL. Blood was drawn and PBMC were isolated as described below.

Ethics approval was granted by the ethics committee of the MRI in accordance with the declaration of Helsinki [586]. Patients were informed about the study goals and their right to withdraw their consent and from being part of it. All patients signed an informed consent form.

### 6.2 PBMC isolation and cryoconservation

Blood was drawn to lithium heparin S-Monovette tubes (Sarstedt, Nümbrecht, Germany) and diluted 1:3 with Phosphate buffered saline (PBS) (Life Technologies, Darmstadt, Germany) and layered on 15 mL Ficoll (human Pancoll, PAN-BIOTECH, Aidenbach,

Germany) in 50 mL Cellstar tubes (Greiner Bio-One, Frickenhausen, Germany). Centrifugation at 800 × g for 20 min at 19 °C without break allowed a gradient separation of the blood cells. Lymphocytes were transferred to 50 mL Cellstar tubes (Greiner Bio-One) with 10 mL PBS.

Alternatively, blood was drawn with the Safety-Lok and Vacutainer system (BD, Franklin Lakes, USA) directly to sodium citrate CPT tubes (BD, Franklin Lakes, USA). Both systems were shown to be comparable regarding viability and T-cell recovery [571-573]. CPT tubes were centrifuged within 2 hours after blood draw (1500 – 1800 × g, 15 min, RT) and sent to the study site in Munich. Lymphocytes and plasma was transferred to a 50 mL Cellstar tube.

Lymphocytes were centrifuged two times at 300 × g for 10 min at 19 °C and washed with 10 mL of PBS. PBMC were resuspended in cryo medium (90 % fetal calf serum (FCS) (Life Technologies, Darmstadt, Germany) and 10 % dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Steinheim, Germany)) at a density of $1 \times 10^7$ cells/mL. Nunc™ Cryovials (Thermo Fisher Scientific, Waltham, USA) were transferred to freezing devices (Mr. Frosty, Thermo Fisher Scientific, Waltham, USA) (precooled to 4 °C), which were moved to -80 °C for 24 – 72 h after isolation. PBMC were eventually stored in the vapor phase of liquid nitrogen tanks.

### 6.3 Cell Counting

PBMC were counted with the automated cell counter ViCell XR (Beckman Coulter, Krefeld, Germany) using trypan blue staining following isolation, thawing and resting.

### 6.4 Thawing and Resting

Cryovials were prewarmed shortly in a bead bath (Memmert, Schwabach, Germany) set to 39 °C until a frozen crystal remained. PBMC were transferred drop-by-drop to 10 mL warm RPMI-10 medium (Roswell Park Memorial Institute medium (RPMI1640) supplemented with 10 % FCS and 1 % penicillin–streptomycin (PenStrep, Life Technologies, Invitrogen, Darmstadt, Germany)), centrifuged (300 × g, 10 min, 19 °C) and washed with RPMI-10 medium two times.

Falcon™ cell strainers (Corning Inc, Corning, USA) with a mesh size of 100 µm were used to obtain a uniform single-cell suspension and PBMC were rested in RPMI-10 medium ($2\times10^6$ PBMC in 1 mL) for 18 h at 37 °C and 5 % $CO_2$.

### 6.5 *In vitro* Stimulation

For stimulation, $1x10^6$ PBMC were seeded to wells of Falcon U-bottom 96-well plates (Fisher Scientific, Hampton, USA) with RPMI-10 + 1 µg/mL α-CD28. 18-mer HBV peptide pools with 11 aminoacids (aa) overlap, spanning the whole sequence of the HBV core, envelope and polymerase proteins (Genaxxon, Ulm, Germany), a HIV-nef ultra peptide pool with 150 15-mer peptides designed to optimally cover the whole HIV-nef protein sequence derived from HIV-1/SIVcpz (JPT Peptide Technologies, Berlin, Germany) and FEC peptide pool with 32 peptides derived from Flu (12 peptides), EBV (15 peptides) and CMV (5 peptides) with a length of 8 – 12 aa that were optimized to challenge CD8 T-cell responses for different HLA types (NIBSC, Potters Bar, UK) [519] were used at concentrations of 2 µM (calculated to a total volume of 200 µL). After 1 h of stimulation, 50 µL of 10 µg/mL brefeldin A (BFA) (Sigma-Aldrich, St. Louis, USA) in RPMI-10 were added to each well. After another 4 h, plates with lid and sealed with parafilm (Bemis, Neenah, US) were transferred to 4 °C over night.

### 6.6 Staining

$1\times10^6$ PBMC in wells of 96-well U-bottom plates were stained with a Live/Dead discriminating dye. Either 50 µL of NIR working solution (1:200 LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit (Life Technologies, Invitrogen, Darmstadt, Germany) or 100 µL of UV Live/Dead working solution (1:100 LIVE/DEAD™ Fixable Blue Dead Cell Stain Kit (Life Technologies, Invitrogen, Darmstadt, Germany) in eBioscience™ Flow Cytometry Staining Buffer (Life Technologies, Invitrogen, Darmstadt, Germany)) was used to determine the vitality of lymphocytes. Live/Dead dye was washed out with Staining Buffer at total volumes of 200 µL (560 × g, 5 min, 4 °C).

Intracellular staining was combined with the surface staining – cells were therefore fixed for 20 min in 100 µL eBioscience™ Intracellular Fixation buffer (Life Technologies, Invitrogen,

Darmstadt, Germany) and afterwards centrifuged (710 × g, 5 min, 4 °C) and washed in 200 µL eBioscience Permeabilization buffer (Life Technologies, Invitrogen, Darmstadt, Germany) for three times. Staining was then proceeded for 30 min with CD3 (BV510, SK7 clone, 1:40) (Biolegend, San Diego, USA), CD4 (PE-CF594, RPA-T4 clone, 1:20), CD8 (PE-Cy7, RPA-T8 clone, 1:200), IL-2 (FITC, MQ1-17H12 clone, 1:32), TNF (eFluor450, Mab11 clone, 1:20) (all eBioscience, Thermo Fisher Scientific, Waltham, USA), IFN-γ (AlexaFluor700, B27 clone, 1:1600) (BD, Franklin Lakes, USA) and unbound dye was again washed out with 200 µL permeabilization buffer (710 × g, 5 min, 4 °C).

For assays with intranuclear stainings, the surface staining of cells was performed first with CD3 (BUV395, SK7 clone, 1:50), CD3 (FITC, SK7 clone, 1:50), CD8 (PE-CF594, RPA-T8 clone, 1:50), CD25 (BV786, MA251 clone, 1:50), CD39 (BV711, TU66 clone, 1:67), CD127 (PE, hIL-7R-M21 clone, 1:80), CCR7 (BB515, 3D12 clone, 1:20) (all BD, Franklin Lakes, USA), CD8 (AlexaFluor700, HIT8a clone, 1:100), CD45RA (BV510, HI100 clone, 1:100), CD127 (BV605, A019D5 clone, 1:50), PD-1 (BV421, EH12.2H7 clone, 1:20) (all Biolegend, San Diego, USA), CD4 (APC, OKT4 clone, 1:25), TIGIT (PE-Cy7, MBSA43 clone, 1:100) (all eBioscience, Thermo Fisher Scientific, Waltham, USA), CD4 (PerCP, M-T466 clone, 1:25) (Miltenyi Biotec, Bergisch Gladbach, Germany) in a total volume of 100 µL for 30 min. Then, cells were fixed for 30 min in 100 µL eBioscience™ FoxP3/Transcription factor Fixation buffer (Life Technologies, Invitrogen, Darmstadt, Germany) and afterwards centrifuged (560 × g, 5 min, 4 °C) and washed in 200 µL eBioscience™ permeabilization buffer (Life Technologies, Invitrogen, Darmstadt, Germany) for three times. Intranuclear staining was then proceeded for 30 min with Tcf-1 (AlexaFluor647, 7F11A10 clone, 1:20) (Biolegend, San Diego, USA), FoxP3 (eFluor450, 236A/E7 clone, 1:33), Eomes (PerCP-e710, WD1928 clone, 1:20), T-bet (PE, eBio4B10 clone, 1:33) (all eBioscience, Thermo Fisher Scientific, Waltham, USA) and unbound dye was again washed out with 100 µL permeabilization buffer (710 × g, 5 min, 4 °C).

For compensation, UltraComp eBeads™ Compensation Beads (Life Technologies, Invitrogen, Darmstadt, Germany) and ArC™ Amine Reactive Compensation Bead Kit (Life Technologies, Invitrogen, Darmstadt, Germany) were stained with antibodies and Live/dead discriminating dyes.

### 6.7 Data acquisition and analysis

After staining, PBMC and compensation samples were transferred to Titertube micro test tubes (Bio-Rad Laboratories, Hercules, USA) and immediately detected at a BD LSRFortessa™ cell analyzer (laser lines: 355 nm, 405 nm, 488 nm, 532 nm, 640 nm) (BD, Franklin Lakes, USA) at low to medium flow rates. Weekly control of the flow cytometer's performance was checked and settings were configured with Cytometer, Setup & Tracking beads (Becton Dickinson, Franklin Lakes, USA).

Raw data of the experiments can be provided per request. Analysis of the results from the flow cytometric assays was performed in FlowJo 10 (FlowJo, LLC, Ashland, Orgeon, USA). Data was quality controlled within the working group. Data for the analysis of polyfunctionality of T cells was preprocessed in Microsoft Excel (Microsoft Corporation, Redmond, USA) and then imported into SPICE 6 (niaid.github.io/spice/). In short, SPICE provides visualization tools for polyfunctionality and cytokine expression profiles of T-cell responses [587].

### 6.8 Statistical analysis

Frequencies of IFN-γ, IL-2 and TNF producing T cells upon peptide pool challenge are given as percentage of the respective T-cell subset. T-cell response data after stimulation was background subtracted with unstimulated T-cell cytokine secretion data of PBMC of the same donor and visit.

Statistical significance was tested with Kruskal-Wallis tests (nonparametric One-way ANOVA), if applicable or two-tailed Mann-Whitney test if required. Correlations were determined on the basis of Spearman's rank correlation coefficient. Statistical analysis was performed in GraphPad Prism 7 (GraphPad Software, San Diego, USA).

### 6.9 Clustering and datamining algorithms

The SPADE algorithm was used to analyze connectivity of cell clusters, their level of T-cell exhaustion marker expression and the relation of the thereby defined subpopulations. We

used RStudio 1.1.463 (RStudio, Boston, USA) to set up the analysis depending on Bendall's and Qiu's instructions [558, 588].

We performed tSNE-based clustering and eventual mapping of marker expressions with the tSNE plugin of FlowJo (Becton Dikinson, Franklin Lakes, USA) [566]. Combinations of 6 steps of iterations (50, 100, 250, 500, 750, 1000) and 3 steps of perplexity (50, 100, 200) were used with an eta learning rate of 21,000. Optimal clustering was found as presented with 1000 iterations, 200 perplexity with the Barnes algorithm.

### 6.10 Lab Environment

The experiments in this study were performed by the same individual trained staff members in our laboratory according to our established SOPs covering the isolation, cryopreservation, storage and thawing of PBMC. Additionally, the stimulation, staining, data acquisition, data processing and gating strategies are defined by our SOPs.

## 7. List of figures & tables

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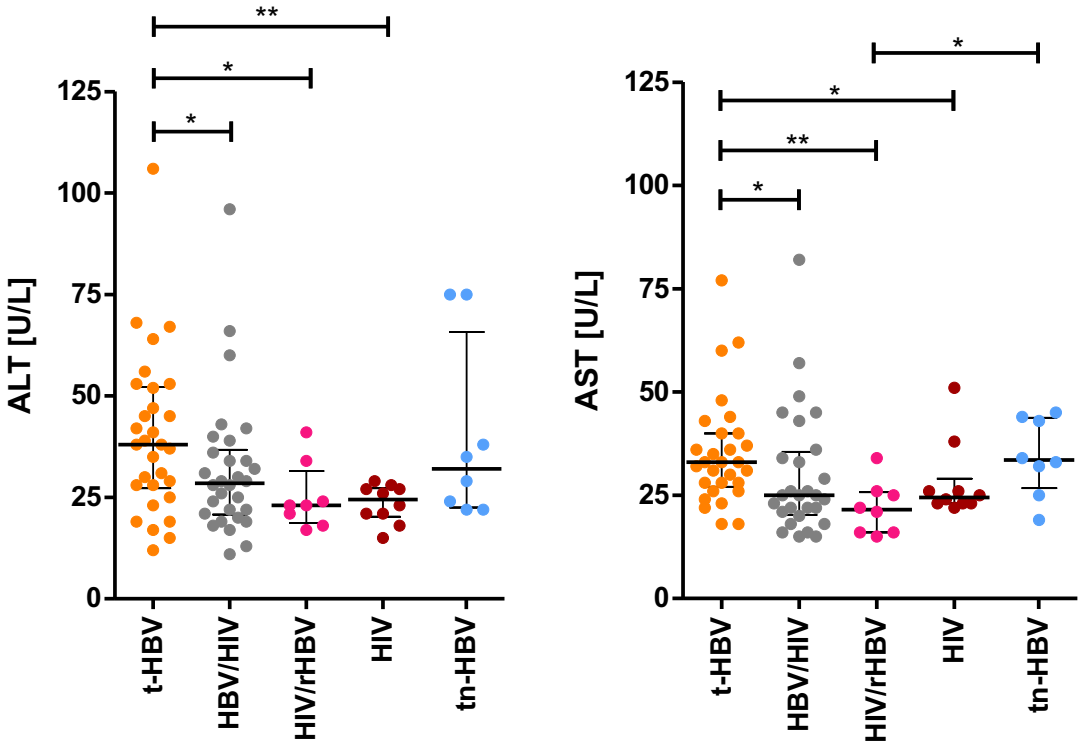
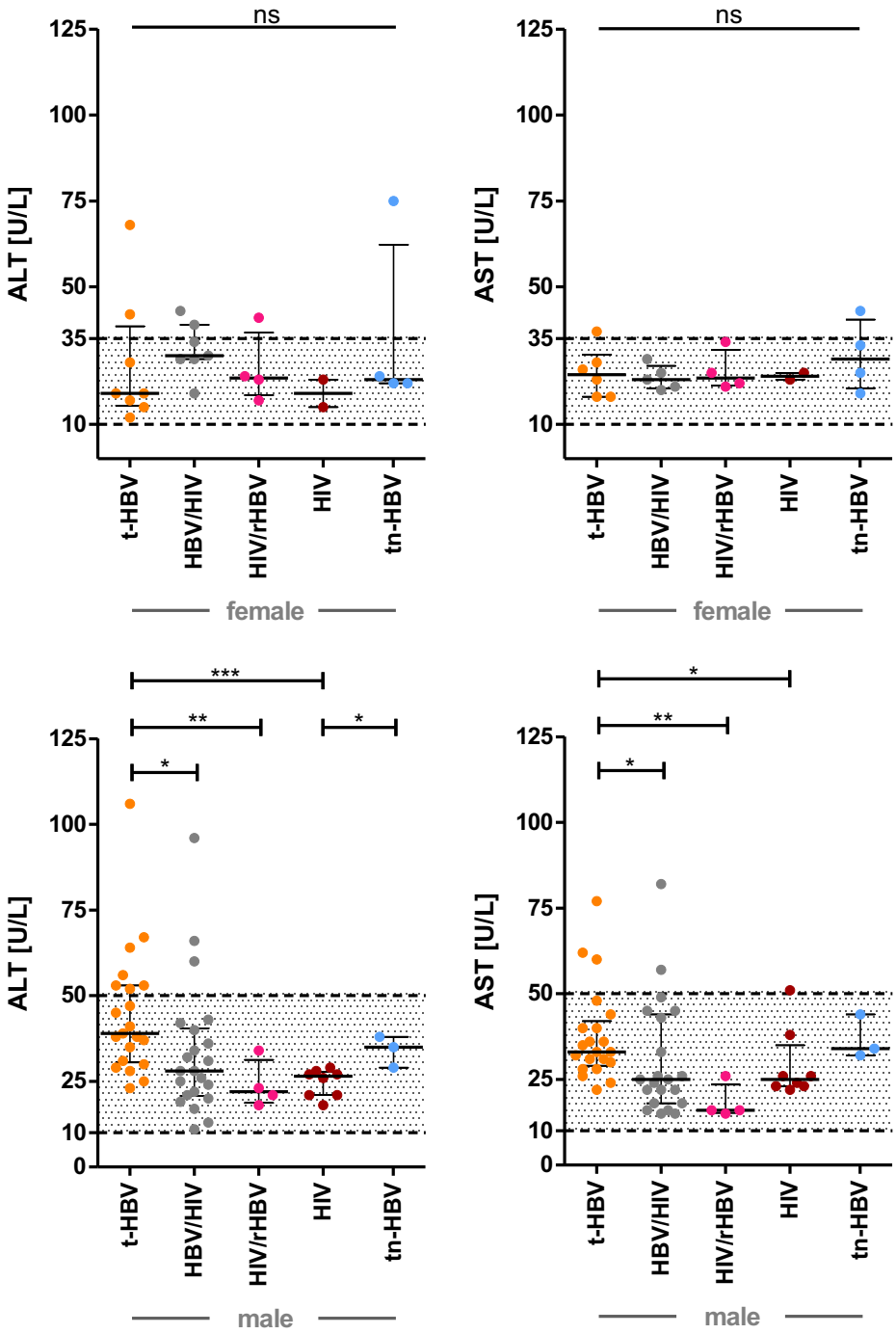
## 9. Supplement

Serum liver enzymes ALT and AST can be used for the detection of inflammation of the liver and liver damage. While elevated ALT and AST levels can be found in patients with pathogenesis like alcohol abuse, HCC or viral hepatitis, an elevation is also found in patients with seroconversion/viral clearance if the immune system depletes infected hepatocytes. Bursts of elevated ALT/AST levels are then referred to as hepatic flares. Norm values for ALT/AST are used in the clinic although they should rather be regarded patient-specific over time. ALT and AST serum levels are shown below (Supplemental Figure 1), while a differentiation for male and female patients is done below (Supplemental Figure 2, Supplemental Table 1).




Supplemental Figure 1: **Serum liver enzyme ALT and AST levels**. Peripheral liver enzyme levels of ALT and AST are displayed for patients with t-HBV (n = 20), HBV/HIV (n = 30), HIV/rHBV (n = 8), HIV (n = 10) or tn-HBV (n = 8) infection. Median values and interquartile (25. and 75. percentile) range are indicated. (Primary Kruskall-Wallis test (ANOVA) with secondary Mann-Whitney U test, ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$)



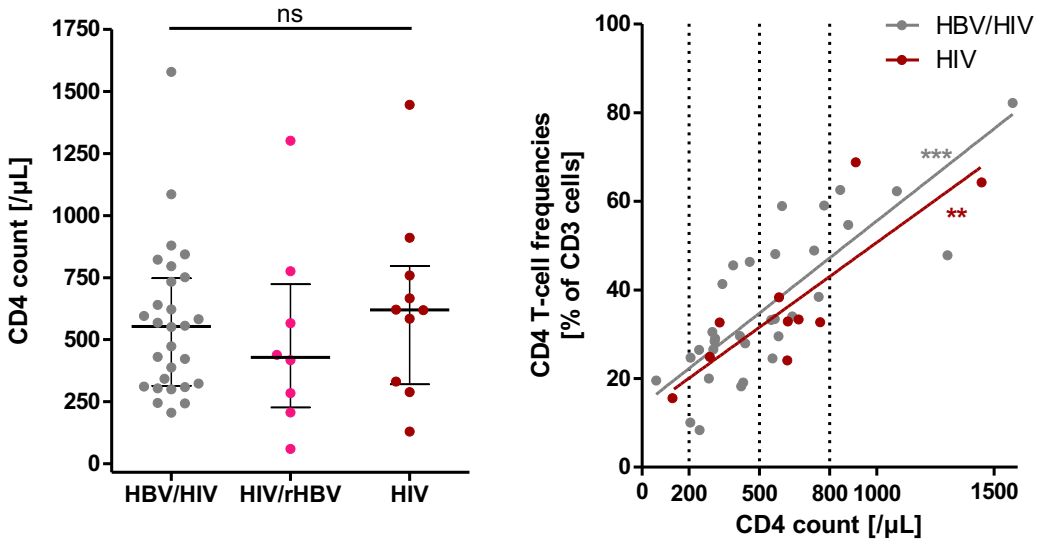

Supplemental Figure 2: **Serum liver enzyme ALT and AST levels of female and male patients**. Peripheral liver enzyme levels of ALT and AST are displayed for tn-HBV (n = 8), t-HBV (n = 20), HBV/HIV (n = 30), HIV/rHBV (n = 8) or HIV (n = 10) patients. Median values and interquartile (25. and 75. percentile) range are indicated. Reference values of ALT and AST are indicated for women 10 – 35 U/L and 10 – 50 U/L for men. (Primary Kruskall-Wallis test (ANOVA) with secondary Mann-Whitney U test, ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$)

Supplemental Table 1: **Serum liver enzyme ALT and AST levels distinguished by gender of the patients.**

| ALT | t-HBV | HBV/HIV | HIV/rHBV | HIV | tn-HBV |
|---|---|---|---|---|---|
| Female | 19 (12-42) | 29.5 (19-39) | 23.5 (17-41) | 19 (15-23) | 23 (22-75) |
| Male | 39 (23-106) | 28 (11-96) | 22 (18-34) | 26.5 (18-29) | 35 (29-38) |

| AST | t-HBV | HBV/HIV | HIV/rHBV | HIV | tn-HBV |
|---|---|---|---|---|---|
| Female | 24.5 (18-37) | 23 (20-29) | 23.5 (21-34) | 24 (23-25) | 29 (19-43) |
| Male | 33 (22-77) | 25 (15-82) | 16 (15-26) | 25 (22-51) | 34 (32-44) |

The immune status of patients can further be analyzed regarding their CD4 T-cell count that was determined from peripheral blood, but also regarding the CD4 T-cell frequency of total CD3 T cells. Since CD4 T cells are depleted in the natural history of HIV/AIDS, but play a major role in HBV-specific immunity, we evaluated the counts and frequencies of CD4 T cells and correlated the results (Supplemental Figure 3).

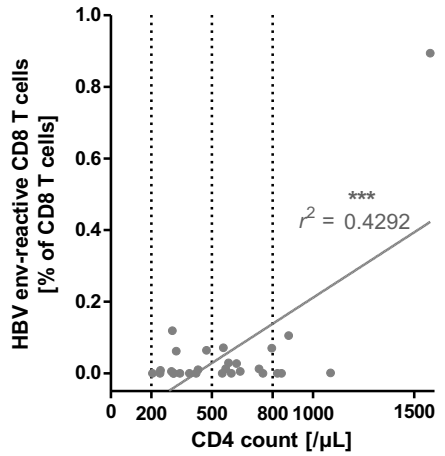



Supplemental Figure 3: **CD4 T-cell counts**/µL were determined for patients with HBV/HIV (n = 30), HIV/rHBV (n = 8) or HIV (n = 10) infection. Correlation of CD4 T-cell counts/µL in whole blood with CD4 T-cell frequency of lymphocytes determined by flow cytometry was performed for patients with HBV/HIV or HIV infection. Median values and interquartile (25. and 75. percentile) range are indicated. (Primary Kruskall-Wallis test (ANOVA); Spearman correlation, ns $p > 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$)

Peripheral CD4 T-cell counts showing patients with HBV/HIV infection to have similar levels of about 500 CD4 cells/µL ($p = 0.512$) as patients with HIV/rHBV or HIV infection. CD4 counts from peripheral blood and CD4 T-cell frequencies after cryo-preservation detected with flow cytometry correlated significantly for patients with HBV/HIV ($r^2 = 0.65$; $p < 0.0001$) or HIV ($r^2 = 0.69$; $p = 0.0029$) infection.

Frequency of HBV env-reactive CD8 T cells of patients with HBV/HIV infection was correlated with peripheral CD4 T-cell counts (Supplemental Figure 4).

HBV env-reactive CD8 T cells correlated strongly ($r^2 = 0.429$) and significantly ($p = 0.0002$) with CD4 T-cell counts. While the significance was robust, the strong positive correlation was mostly dependent on the data of one patient (Bn23). Neither HBV core-, nor pol-reactive T-cell frequencies correlated with CD4 counts (not shown).




Supplemental Figure 4: **Correlation of HBV env-reactive CD8 T cells with CD4 T-cell counts.** CD4 T-cell counts/µL were correlated with HBV env-reactive CD8 T-cell frequencies of patients with HBV/HIV infection (n = 30), Spearman correlation, ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$)

The sequence differences of HBV genotypes with each other were assessed to analyse a possible influence of the underlying genotype of the used HBV protein-derived peptide pools on the stimulation of PBMC of the different cohorts.

Supplemental Table 2: **Comparison of HBV Proteins Genotype B and D**

| Protein | AA Size (B vs D) | Score | Identities | Positives | Gaps |
|---|---|---|---|---|---|
| Core | 212 | 354 | 95% | 97% | 0% |
| HBs | 400 vs 389 | 654 | 88% | 92% | 0% |
| Pol | 843 vs 832 | 1417 | 85% | 91% | 1% |
| X | 154 | 277 | 88% | 90% | 0% |

Supplemental Table 3: **Comparison of HBV Proteins Genotype C and D**

| Protein | AA Size (C vs D) | Score | Identities | Positives | Gaps |
|---|---|---|---|---|---|
| Core | 212 | 352 | 95% | 97% | 0% |
| HBs | 400 vs 389 | 655 | 90% | 93% | 0% |
| Pol | 843 vs 832 | 1484 | 87% | 92% | 1% |
| X | 154 | 291 | 93% | 94% | 0% |

Supplemental Table 4: **Comparison of HBV Proteins Genotype C and B**

| Protein | AA Size (C vs B) | Score | Identities | Positives | Gaps |
|---|---|---|---|---|---|
| Core | 183 | 288 | 91% | 96% | 0% |
| HBs | 400 | 659 | 86% | 92% | 0% |
| Pol | 843 | 1514 | 88% | 93% | 0% |
| X | 154 | 276 | 88% | 91% | 0% |

Supplemental Table 5: **Comparison of HBV Proteins Genotype A and B**

| Protein | AA Size (A vs B) | Score | Identities | Positives | Gaps |
|---|---|---|---|---|---|
| Core | 185 vs 183 | 297 | 95% | 97% | 0% |
| HBs | 400 | 673 | 88% | 93% | 0% |
| Pol | 845 vs 843 | 1486 | 88% | 92% | 0% |
| X | 154 | 275 | 88% | 91% | 0% |

Supplemental Table 6: **Comparison of HBV Proteins Genotype A and C**

| Protein | AA Size (A vs C) | Score | Identities | Positives | Gaps |
|---|---|---|---|---|---|
| Core | 214 vs 212 | 351 | 94% | 98% | 0% |
| HBs | 400 | 676 | 90% | 93% | 0% |
| Pol | 845 vs 843 | 1538 | 89% | 94% | 0% |
| X | 154 | 286 | 92% | 94% | 0% |

Supplemental Table 7: **Comparison of HBV Proteins Genotype A and D**

| Protein | AA Size (A vs D) | Score | Identities | Positives | Gaps |
|---|---|---|---|---|---|
| Core | 214 vs 212 | 416 | 96% | 97% | 0% |
| HBs | 400 vs 389 | 632 | 87% | 91% | 0% |
| Pol | 845 vs 832 | 1444 | 85% | 90% | 1% |
| X | 154 | 295 | 95% | 96% | 0% |

Supplemental Table 8: **Comparison of HBV Proteins Genotype A and E**

| Protein | AA Size (A vs E) | Score | Identities | Positives | Gaps |
|---|---|---|---|---|---|
| Core | 214 vs 212 | 356 | 96% | 98% | 0% |
| HBs | 400 vs 399 | 631 | 86% | 91% | 0% |
| Pol | 845 vs 842 | 1456 | 86% | 91% | 0% |
| X | 154 | 288 | 94% | 95% | 0% |

Supplemental Table 9: **Comparison of HBV Proteins Genotype B and E**

| Protein | AA Size (B vs E) | Score | Identities | Positives | Gaps |
|---|---|---|---|---|---|
| Core | 183 | 289 | 91% | 95% | 0% |
| HBs | 400 vs 399 | 627 | 85% | 90% | 0% |
| Pol | 843 vs 842 | 1427 | 84% | 90% | 0% |
| X | 154 | 274 | 87% | 91% | 0% |

Supplemental Table 10: **Comparison of HBV Proteins Genotype C and E**

| Protein | AA Size (C vs E) | Score | Identities | Positives | Gaps |
|---|---|---|---|---|---|
| Core | 212 | 350 | 94% | 96% | 0% |
| HBs | 400 vs 399 | 652 | 90% | 93% | 0% |
| Pol | 843 vs 842 | 1479 | 86% | 92% | 0% |
| X | 154 | 288 | 93% | 94% | 0% |

Supplemental Table 11: **Comparison of HBV Proteins Genotype D and E**

| Protein | AA Size (D vs E) | Score | Identities | Positives | Gaps |
|---|---|---|---|---|---|
| Core | 212 | 426 | 98% | 98% | 0% |
| HBs | 389 vs 399 | 660 | 90% | 95% | 0% |
| Pol | 832 vs 842 | 1503 | 88% | 93% | 1% |
| X | 154 | 301 | 97% | 96% | 0% |

Peptide pools were used to stimulate T-cell responses as described in 6.5. The following Supplemental tables 12 – 14 show the peptides used in the HBV protein-derived peptide pools, as well as the FEC peptide pool (Supplemental table 15)

Supplemental Table 12: **Peptides in the used HBV pol-derived peptide pool**

| Peptide | Sequence | Ag |
|---|---|---|
| HBV1 | MPLSYQHFRRLLLLDDEA | Pol |
| HBV2 | FRRLLLLDDEAGPLEEEL | Pol |
| HBV3 | DDEAGPLEEELPRLADEG | Pol |
| HBV4 | EEELPRLADEGLNRRVAE | Pol |
| HBV5 | ADEGLNRRVAEDLNLGNL | Pol |
| HBV6 | RVAEDLNLGNLNVSIPWT | Pol |
| HBV7 | LGNLNVSIPWTHKVGNFT | Pol |
| HBV8 | IPWTHKVGNFTGLYSSTV | Pol |
| HBV9 | GNFTGLYSSTVPVFNPHW | Pol |
| HBV10 | SSTVPVFNPHWKTPSFPN | Pol |
| HBV11 | NPHWKTPSFPNIHLHQDI | Pol |
| HBV12 | SFPNIHLHQDIIKKCEQF | Pol |
| HBV13 | HQDIIKKCEQFVGPLTVN | Pol |
| HBV14 | CEQFVGPLTVNEKRRLQL | Pol |
| HBV15 | LTVNEKRRLQLIMPARFY | Pol |
| HBV16 | RLQLIMPARFYPNVTKYL | Pol |
| HBV17 | ARFYPNVTKYLPLDKGIK | Pol |
| HBV18 | TKYLPLDKGIKPYYPEHL | Pol |
| HBV19 | KGIKPYYPEHLVNHYFQT | Pol |
| HBV20 | PEHLVNHYFQTRHYLHTL | Pol |
| HBV21 | YFQTRHYLHTLWKAGILY | Pol |
| HBV22 | LHTLWKAGILYKRETTHS | Pol |
| HBV23 | GILYKRETTHSASFCGSP | Pol |
| HBV24 | TTHSASFCGSPYSWEQEL | Pol |
| HBV25 | CGSPYSWEQELQHGAESF | Pol |
| HBV26 | EQELQHGAESFHQQSSGI | Pol |

| | | |
|---|---|---|
| HBV27 | AESFHQQSSGILSRPPVG | Pol |
| HBV28 | SSGILSRPPVGSSLQSKH | Pol |
| HBV29 | PPVGSSLQSKHRKSRLGL | Pol |
| HBV30 | QSKHRKSRLGLQSQQGHL | Pol |
| HBV31 | RLGLQSQQGHLARRQQGR | Pol |
| HBV32 | QGHLARRQQGRSWSIRAG | Pol |
| HBV33 | QQGRSWSIRAGIHPTARR | Pol |
| HBV34 | IRAGIHPTARRPFGVEPS | Pol |
| HBV35 | TARRPFGVEPSGSGHTTN | Pol |
| HBV36 | VEPSGSGHTTNLASKSAS | Pol |
| HBV37 | HTTNLASKSASCLYQSPV | Pol |
| HBV38 | KSASCLYQSPVRKAAYPA | Pol |
| HBV39 | QSPVRKAAYPAVSTFEKH | Pol |
| HBV40 | AYPAVSTFEKHSSSGHAV | Pol |
| HBV41 | FEKHSSSGHAVELHNLPP | Pol |
| HBV42 | GHAVELHNLPPNSARSQS | Pol |
| HBV43 | NLPPNSARSQSERPVFPC | Pol |
| HBV44 | RSQSERPVFPCWWLQFRN | Pol |
| HBV45 | VFPCWWLQFRNSKPCSDY | Pol |
| HBV46 | QFRNSKPCSDYCLSHIVN | Pol |
| HBV47 | CSDYCLSHIVNLLEDWGP | Pol |
| HBV48 | HIVNLLEDWGPCAEHGEH | Pol |
| HBV49 | DWGPCAEHGEHHIRIPRT | Pol |
| HBV50 | HGEHHIRIPRTPARVTGG | Pol |
| HBV51 | IPRTPARVTGGVFLVDKN | Pol |
| HBV52 | VTGGVFLVDKNPHNTAES | Pol |
| HBV53 | VDKNPHNTAESRLVVDFS | Pol |
| HBV54 | TAESRLVVDFSQFSRGNY | Pol |
| HBV55 | VDFSQFSRGNYRVSWPKF | Pol |
| HBV56 | RGNYRVSWPKFAVPNLQS | Pol |
| HBV57 | WPKFAVPNLQSLTNLLSS | Pol |
| HBV58 | NLQSLTNLLSSNLSWLSL | Pol |
| HBV59 | LLSSNLSWLSLDVSAAFY | Pol |

| | | |
|---|---|---|
| HBV60 | WLSLDVSAAFYHLPLHPA | Pol |
| HBV61 | AAFYHLPLHPAAMPHLLV | Pol |
| HBV62 | LHPAAMPHLLVGSSGLSR | Pol |
| HBV63 | HLLVGSSGLSRYVARLSS | Pol |
| HBV64 | GLSRYVARLSSNSRIFNH | Pol |
| HBV65 | RLSSNSRIFNHQHGTMQN | Pol |
| HBV66 | IFNHQHGTMQNLHDSCSR | Pol |
| HBV67 | TMQNLHDSCSRNLYVSLL | Pol |
| HBV68 | SCSRNLYVSLLLLYQTFG | Pol |
| HBV69 | VSLLLLYQTFGRKLHLYS | Pol |
| HBV70 | QTFGRKLHLYSHPIILGF | Pol |
| HBV71 | HLYSHPIILGFRKIPMGV | Pol |
| HBV72 | ILGFRKIPMGVGLSPFLL | Pol |
| HBV73 | PMGVGLSPFLLAQFTSAI | Pol |
| HBV74 | PFLLAQFTSAICSVVRRA | Pol |
| HBV75 | TSAICSVVRRAFPHCLAF | Pol |
| HBV76 | VRRAFPHCLAFSYMDDVV | Pol |
| HBV77 | CLAFSYMDDVVLGAKSVQ | Pol |
| HBV78 | DDVVLGAKSVQHLESLFT | Pol |
| HBV79 | KSVQHLESLFTAVTNFLL | Pol |
| HBV80 | SLFTAVTNFLLSLGIHLN | Pol |
| HBV81 | NFLLSLGIHLNPNKTKRW | Pol |
| HBV82 | IHLNPNKTKRWGYSLHFM | Pol |
| HBV83 | TKRWGYSLHFMGYVIGCY | Pol |
| HBV84 | LHFMGYVIGCYGSLPQDH | Pol |
| HBV85 | IGCYGSLPQDHIIQKIKE | Pol |
| HBV86 | PQDHIIQKIKECFRKLPV | Pol |
| HBV87 | KIKECFRKLPVNRPIDWK | Pol |
| HBV88 | KLPVNRPIDWKVCQRIVG | Pol |
| HBV89 | IDWKVCQRIVGLLGFAAP | Pol |
| HBV90 | RIVGLLGFAAPFTQCGYP | Pol |
| HBV91 | FAAPFTQCGYPALMPLYA | Pol |
| HBV92 | CGYPALMPLYACIQSKQA | Pol |

| | | |
|---|---|---|
| HBV93 | PLYACIQSKQAFTFSPTY | Pol |
| HBV94 | SKQAFTFSPTYKAFLCKQ | Pol |
| HBV95 | SPTYKAFLCKQYLNLYPV | Pol |
| HBV96 | LCKQYLNLYPVARQRPGL | Pol |
| HBV97 | LYPVARQRPGLCQVFADA | Pol |
| HBV98 | RPGLCQVFADATPTGWGL | Pol |
| HBV99 | FADATPTGWGLVMGHQRM | Pol |
| HBV100 | GWGLVMGHQRMRGTFLAP | Pol |
| HBV101 | HQRMRGTFLAPLPIHTAE | Pol |
| HBV102 | FLAPLPIHTAELLAACFA | Pol |
| HBV103 | HTAELLAACFARSRSGAN | Pol |
| HBV104 | ACFARSRSGANILGTDNS | Pol |
| HBV105 | SGANILGTDNSVVLSRKY | Pol |
| HBV106 | TDNSVVLSRKYTSFPWLL | Pol |
| HBV107 | SRKYTSFPWLLGCAANWI | Pol |
| HBV108 | PWLLGCAANWILRGTSFV | Pol |
| HBV109 | ANWILRGTSFVYVPSALN | Pol |
| HBV110 | TSFVYVPSALNPADDPSR | Pol |
| HBV111 | SALNPADDPSRGRLGLSR | Pol |
| HBV112 | DPSRGRLGLSRPLLRLPF | Pol |
| HBV113 | GLSRPLLRLPFRPTTGRT | Pol |
| HBV114 | RLPFRPTTGRTSLYADSP | Pol |
| HBV115 | TGRTSLYADSPSVPSHLP | Pol |
| HBV116 | ADSPSVPSHLPDRVHFAS | Pol |
| HBV117 | SHLPDRVHFASPLHVAWR | Pol |
| HBV118 | HFASPLHVAWRPP | Pol |
| HBV119 | LPDRVHFASPLHVAWRPP | Pol |

Supplemental Table 13: **Peptides in the used HBV env-derived peptide pool**

| | | |
|---|---|---|
| HBV120 | MGQNLSTSNPLGFFPDHQ | PreS |
| HBV121 | SNPLGFFPDHQLDPAFRA | PreS |
| HBV122 | PDHQLDPAFRANTANPDW | PreS |
| HBV123 | AFRANTANPDWDFNPNKD | PreS |
| HBV124 | NPDWDFNPNKDTWPDANK | PreS |
| HBV125 | PNKDTWPDANKVGAGAFG | PreS |
| HBV126 | DANKVGAGAFGLGFTPPH | PreS |
| HBV127 | GAFGLGFTPPHGGLLGWS | PreS |
| HBV128 | TPPHGGLLGWSPQAQGIL | PreS |
| HBV129 | LGWSPQAQGILQTLPANP | PreS |
| HBV130 | QGILQTLPANPPPASTNR | PreS |
| HBV131 | PANPPPASTNRQSGRQPT | PreS |
| HBV132 | STNRQSGRQPTPLSPPLR | PreS |
| HBV133 | RQPTPLSPPLRNTHPQAM | PreS |
| HBV134 | PPLRNTHPQAMQWNSTTF | PreS |
| HBV135 | PQAMQWNSTTFHQTLQDP | PreS |
| HBV136 | STTFHQTLQDPRVRGLYF | PreS |
| HBV137 | LQDPRVRGLYFPAGGSSS | PreS |
| HBV138 | GLYFPAGGSSSGTVNPVP | PreS |
| HBV139 | GSSSGTVNPVPTTVSPIS | PreS |
| HBV140 | NPVPTTVSPISSIFSRIG | PreS |
| HBV141 | SPISSIFSRIGDPALNME | PreS |
| HBV142 | SRIGDPALNMENITSGFL | S |
| HBV143 | LNMENITSGFLGPLLVLQ | S |
| HBV144 | SGFLGPLLVLQAGFFLLT | S |
| HBV145 | LVLQAGFFLLTRILTIPQ | S |
| HBV146 | FLLTRILTIPQSLDSWWT | S |
| HBV147 | TIPQSLDSWWTSLNFLGG | S |
| HBV148 | SWWTSLNFLGGTTVCLGQ | S |
| HBV149 | FLGGTTVCLGQNSQSPTS | S |
| HBV150 | CLGQNSQSPTSNHSPTSC | S |
| HBV151 | SPTSNHSPTSCPPTCPGY | S |

| | | |
|---|---|---|
| HBV152 | PTSCPPTCPGYRWMCLRR | S |
| HBV153 | CPGYRWMCLRRFIIFLFI | S |
| HBV154 | CLRRFIIFLFILLLCLIF | S |
| HBV155 | FLFILLLCLIFLLVLLDY | S |
| HBV156 | CLIFLLVLLDYQGMLPVC | S |
| HBV157 | LLDYQGMLPVCPLIPGSS | S |
| HBV158 | LPVCPLIPGSSTTSTGPC | S |
| HBV159 | PGSSTTSTGPCRTCTTPA | S |
| HBV160 | TGPCRTCTTPAQGTSMYP | S |
| HBV161 | TTPAQGTSMYPSCCCTKP | S |
| HBV162 | SMYPSCCCTKPSDGNCTC | S |
| HBV163 | CTKPSDGNCTCIPIPSSW | S |
| HBV164 | NCTCIPIPSSWAFGKFLW | S |
| HBV165 | PSSWAFGKFLWEWASARF | S |
| HBV166 | KFLWEWASARFSWLSLLV | S |
| HBV167 | SARFSWLSLLVPFVQWFV | S |
| HBV168 | SLLVPFVQWFVGLSPTVW | S |
| HBV169 | QWFVGLSPTVWLSVIWMM | S |
| HBV170 | PTVWLSVIWMMWYWGPSL | S |
| HBV171 | IWMMWYWGPSLYSILSPF | S |
| HBV172 | GPSLYSILSPFLPLLPIF | S |
| HBV173 | LSPFLPLLPIFFCLWVYI | S |

Supplemental Table 14: **Peptides in the used HBV core-derived peptide pool**

| | | |
|---|---|---|
| HBV174 | MQLFHLCLIISCSCPTVQ | Core/PreCore |
| HBV175 | LIISCSCPTVQASKLCLG | Core/PreCore |
| HBV176 | PTVQASKLCLGWLWGMDI | Core/PreCore |
| HBV177 | LCLGWLWGMDIDPYKEFG | Core/PreCore |
| HBV178 | GMDIDPYKEFGATVELLS | Core/PreCore |
| HBV179 | KEFGATVELLSFLPSDFF | Core/PreCore |
| HBV180 | ELLSFLPSDFFPSVRDLL | Core/PreCore |
| HBV181 | SDFFPSVRDLLDTASALY | Core/PreCore |
| HBV182 | RDLLDTASALYREALESP | Core/PreCore |

| | | |
|---|---|---|
| HBV183 | SALYREALESPEHCSPHH | Core/PreCore |
| HBV184 | LESPEHCSPHHTALRQAI | Core/PreCore |
| HBV185 | SPHHTALRQAILCWGELM | Core/PreCore |
| HBV186 | RQAILCWGELMTLATWVG | Core/PreCore |
| HBV187 | GELMTLATWVGGNLEDPI | Core/PreCore |
| HBV188 | TWVGGNLEDPISRDLVVS | Core/PreCore |
| HBV189 | EDPISRDLVVSYVNTNMG | Core/PreCore |
| HBV190 | LVVSYVNTNMGLKFRQLL | Core/PreCore |
| HBV191 | TNMGLKFRQLLWFHISCL | Core/PreCore |
| HBV192 | RQLLWFHISCLTFGRETV | Core/PreCore |
| HBV193 | ISCLTFGRETVIEYLVSF | Core/PreCore |
| HBV194 | RETVIEYLVSFGVWIRTP | Core/PreCore |
| HBV195 | LVSFGVWIRTPPAYRPPN | Core/PreCore |
| HBV196 | IRTPPAYRPPNAPILSTL | Core/PreCore |
| HBV197 | RPPNAPILSTLPETTVVR | Core/PreCore |
| HBV198 | LSTLPETTVVRRRGRSPR | Core/PreCore |
| HBV199 | TVVRRRGRSPRRRTPSPR | Core/PreCore |
| HBV200 | RSPRRRTPSPRRRRSQSP | Core/PreCore |
| HBV201 | PSPRRRRSQSPRRRRSQS | Core/PreCore |
| HBV202 | SQSPRRRRSQSRESQC | Core/PreCore |
| HBV203 | RRSQSPRRRRSQSRESQC | Core/PreCore |

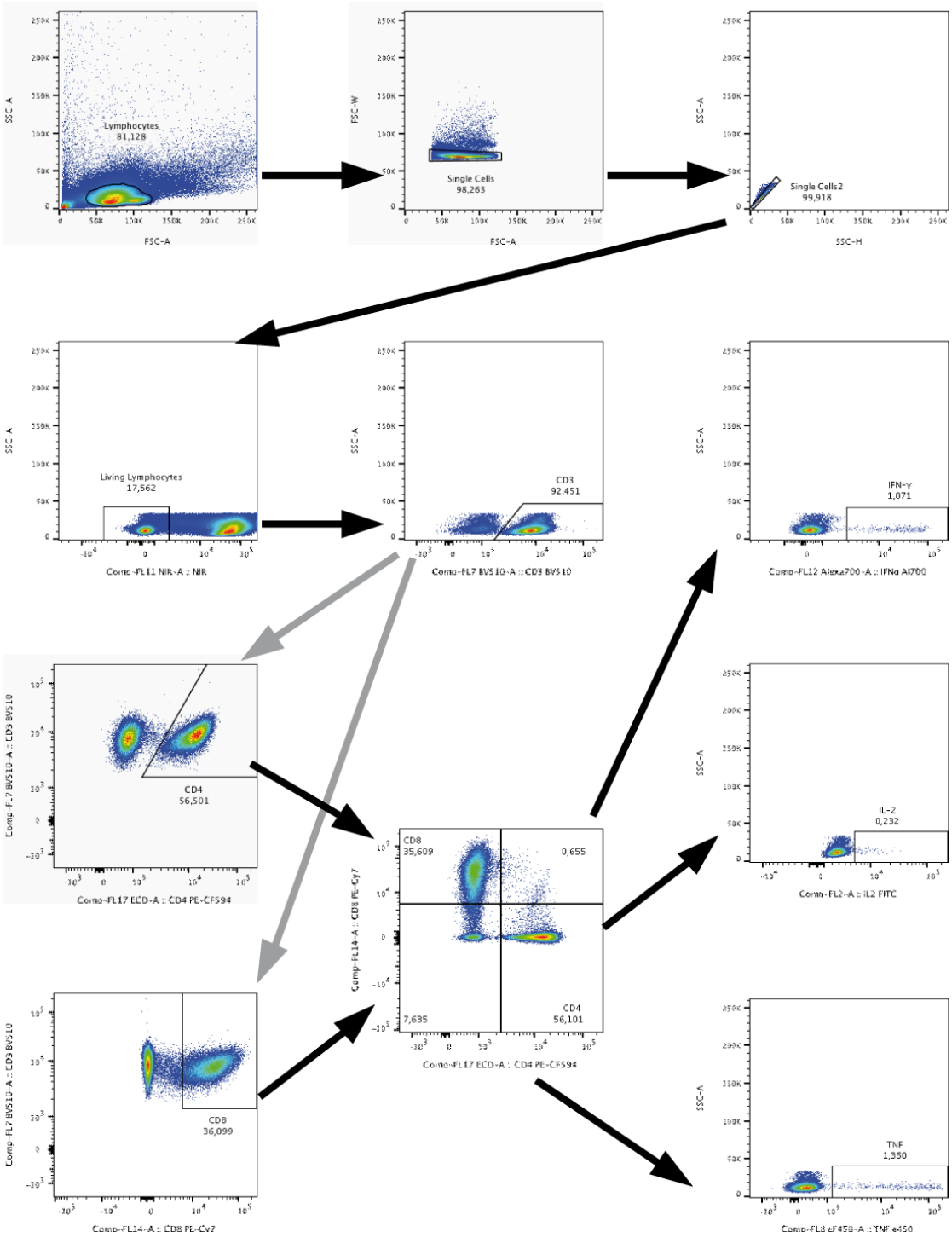
Supplemental Table 15: **Peptides in the used FEC-derived peptide pool**

| Peptide | HLA allele | Sequence | Antigen | Cox |
|---|---|---|---|---|
| FEC1 | A2 | GILGFVFTL | Flu M | + |
| FEC2 | A2 | FMYSDFHFI | Flu | |
| FEC3 | A3/11/6081 | SIIPSGPLK | Flu M | |
| FEC4 | A3 | RVLSFIKGTK | Flu NP | |
| FEC5 | A68 | KTGGPIYKR | Flu NP | + |
| FEC6 | B7 | LPFDKTTVM | Flu NP | |
| FEC7 | B8 | ELRSRYWAI | Flu NP | + |
| FEC8 | B27 | SRYWAIRTE | Flu NP | + |
| FEC9 | B27 | ASCMGLIY | Flu M | |

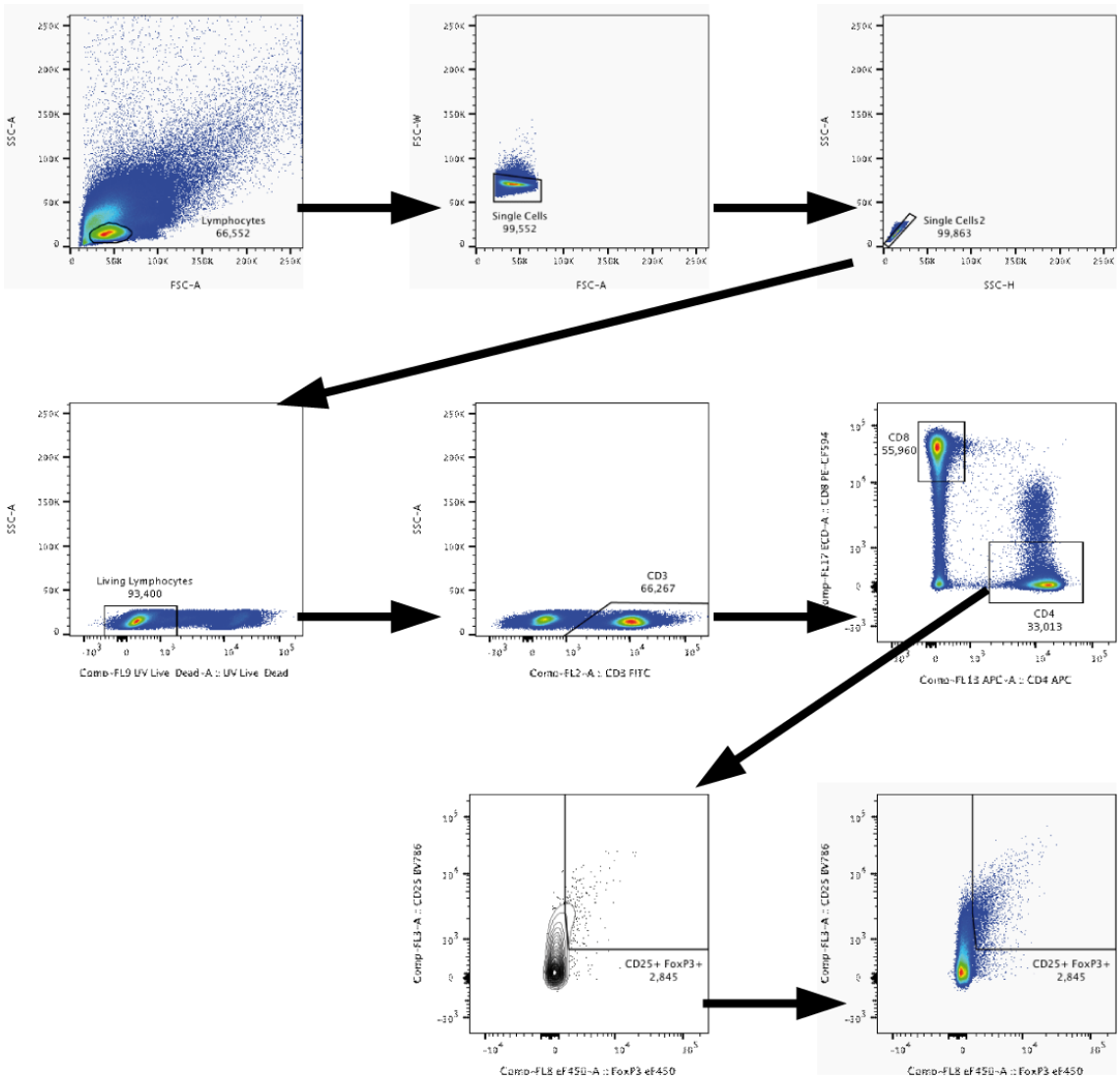
| | | | | |
|---|---|---|---|---|
| FEC10 | A2 | CLGGLLTMV | EBV | |
| FEC11 | A2 | GLCTLVAML | EBV | + |
| FEC12 | A11 | AVFDRKSDAK | EBV | |
| FEC14 | A24 | DYCNVLNKEF | EBV | + |
| FEC15 | B8 | RAKFKQLL | EBV | + |
| FEC16 | B8 | FLRGRAYGL | EBV | + |
| FEC17 | B8 | QAKWRLQTL | EBV | + |
| FEC18 | B27 | RRIYDLIEL | EBV | + |
| FEC19 | B35 | YPLHEQHGM | EBV | + |
| FEC20 | A0201 | NLVPMVATV | CMV | + |
| FEC21 | B0702 | TPRVTGGGAM | CMV | + |
| FEC22 | B18 | SDEEEAIVAYTL | CMV | |
| FEC23 | B35 | IPSINVHHY | CMV | |
| FEC24 | A1 | VSDGGPNLY | Flu | + |
| FEC25 | A1 | CTELKLSDY | Flu | + |
| FEC26 | A3 | ILRGSVAHK | Flu | + |
| FEC27 | A3 | RVRAYTYSK | EBV | + |
| FEC28 | A3 | RLRAEAQVK | EBV | + |
| FEC29 | A11 | IVTDFSVIK | EBV | + |
| FEC30 | A11 | ATIGTAMYK | EBV | + |
| FEC31 | B7 | RPPIFIRRL | EBV | + |
| FEC32 | B44 | EENLLDFVRF | EBV | + |
| FEC33 | B44 | EFFWDANDIY | CMV | + |

T-cell responses were determined with flow cytometry-based ICS, whereas both regulatory T cells and T-cell exhaustion were detected after intranuclear staining and flow cytometric analysis. The staining was performed as described in 6.6.

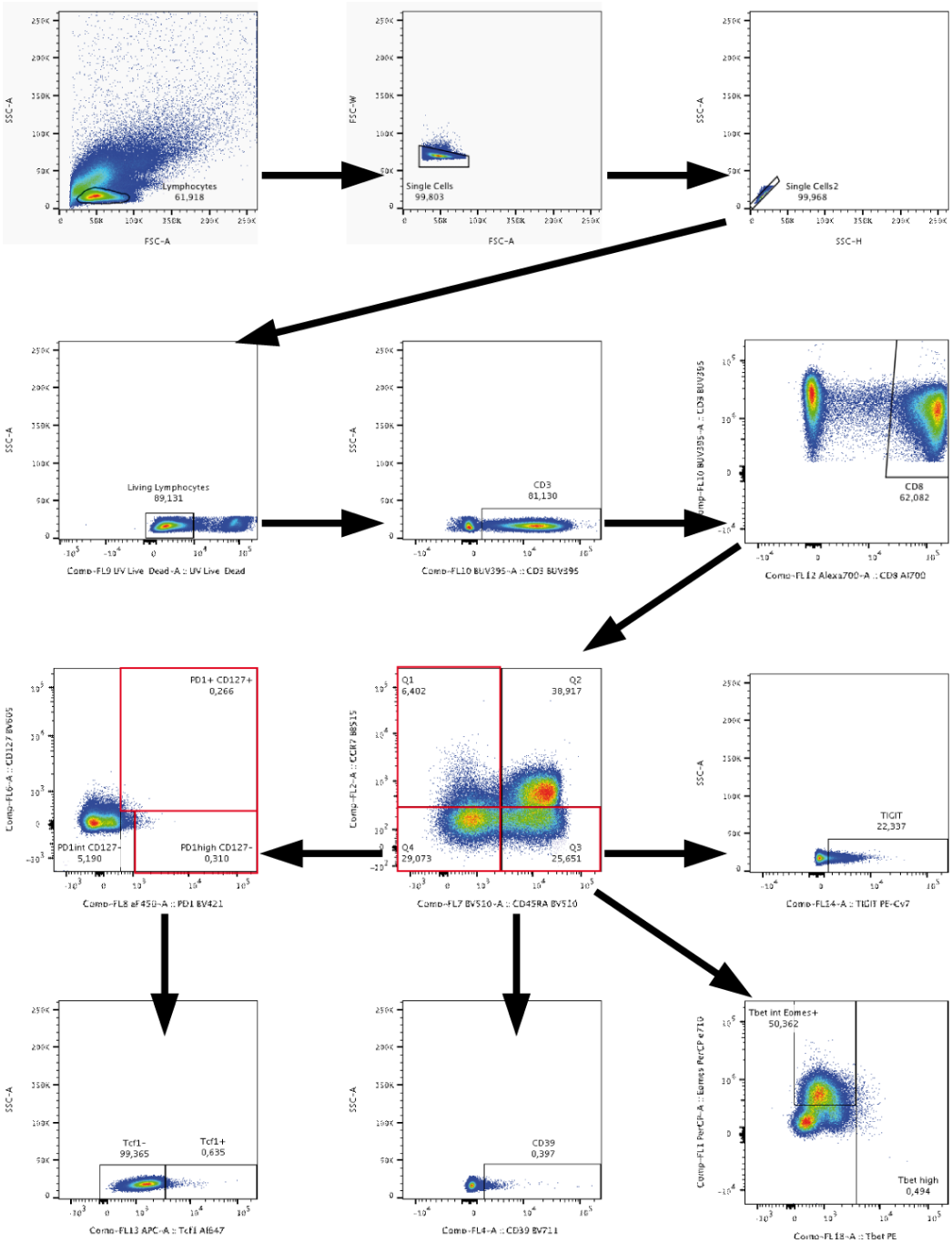
Resulting data was analyzed in FlowJo as described in 6.7. The gating strategies for the T-cell response panel (Supplemental Figure 5), the Treg panel (Supplemental Figure 6) and the T-cell exhaustion panel (Supplemental Figure 7) are shown below.




Supplemental figure 5: **Gating strategy for the detection of T-cell responses.** Lymphocytes were gated in FCS-A vs. SSC-A. The resulting population was gated in FSC-A vs. FSC-W and SSC-H vs. SSC-A for singlets. Living lymphocytes were identified as cells that show no signal for the Live/Dead cells discriminating dye. CD3 cells were gated and CD4+ and CD8+ T-cell populations thereof. From the CD4 and CD8 gating, a Boolean combined gating was performed identifying CD4+ CD8- and CD4- CD8+ T cells. Cytokine producing T cells were determined by gating on IFN-, IL-2 and TNF individually for both CD4 and CD8 T cells respectively. Combination Boolean gates for cytokine producing polyfunctional T cells were calculated. Plots are depicted as pseudocolours. Pictures are representative examples of the respective gating in this panel.




Supplemental figure 6: **Gating strategy for the detection of regulatory T cells.** Lymphocytes were gated in FCS-A vs. SSC-A. The resulting population was gated in FSC-A vs. FSC-W and SSC-H vs. SSC-A for singlets. Living lymphocytes were identified as cells that show no signal for the Live/Dead cells discriminating dye. CD3 cells were gated and CD4+ CD8- T cells thereof. CD25+ FoxP3+ T cells were determined in the CD4 T-cell population with the help of FMO controls. Plots are depicted as pseudocolours, whereas the CD25+ FoxP3+ population is also shown as a contour plot. Pictures are representative examples of the respective gating in this panel.




Supplemental figure 7: **Gating strategy for the detection of T-cell exhaustion.** Lymphocytes were gated in FCS-A vs. SSC-A. The resulting population was gated in FSC-A vs. FSC-W and SSC-H vs. SSC-A for singlets. Living lymphocytes were identified as cells that show no signal for the Live/Dead cells discriminating dye. CD3 cells were gated and CD8+ T cells thereof. Subsets of naïve, central memory, effector memory and terminal effector memory T cells were identified with CD45RA vs. CCR7 gating. Exhaustion marker expression was quantified on non-naïve CD8 T cells. The exhaustion surface markers TIGIT and CD39 were gated in relation to an FMO control. Combinations of the transcription factors T-bet and Eomes expression were distinguished with the help of FMO controls. Subpopulations of PD-1 and CD127 producing CD8 T cells were identified with FMO controls, which were afterwards explored regarding the transcription factor tcf-1 expression. Plots are depicted as pseudocolours. Pictures are representative examples of the respective gating in this panel.

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## Publications and Meetings

‚Altered hepatitis B virus-specific T-cell immunity in hepatitis B virus and human immunodeficiency virus 1 co- versus hepatitis B virus mono-infected patients '
(Feuerherd, M; Körber, N; Boesecke, C; Mohrmann, K; Fröschl, G; Ringelhan, M; Neumann-Haefelin, C; Spinner, CD; Geisler, F; Rockstroh, JK; Protzer, U; Bauer, T; *Hepatology*, in preparation)

‚Altered HBV-specific T-cell immunity in HBV/HIV-1 co- versus HBV mono-infected patients'
(Feuerherd, M; Körber, N; Boesecke, C; Mohrmann, K; Fröschl, G; Ringelhan, M; Neumann-Haefelin, C; Spinner, CD; Geisler, F; Rockstroh, JK; Protzer, U; Bauer, T; *International HBV Meeting*, Oct 2019)

‚Altered HBV-specific CD4+ T-cell Responses in HBV/HIV-1 Co- versus HBV Mono-infected Patients'
(Feuerherd, M; Körber, N; Boesecke, C; Mohrmann, K; Fröschl, G; Ringelhan, M; Geisler, F; Rockstroh, J; Protzer, U; Bauer, T; *Journal of Viral Hepatitis (Global Hepatitis Summit)*, vol 25, s2, Jun 2018; DOI: 10.1111/jvh.34_12923)