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IMPAIRED RESPONSE TO HBV VACCINATION IN HIV-1 INFECTED CHILDREN: IMMUNOPATHOLOGICAL MECHANISMS

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Impaired response to HBV vaccination in HIV-1 infected children: Immunopathological mechanisms

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my mother Bekelech Engedaget Worku

“የጌታ ፈቃድ ይሁን ብለን ዝም አልን”

“we ceased, saying, The will of the Lord will be done”

ABSTRACT

HBV vaccination prevents HBV infection and related liver cancer. Immunological dysfunctions of Tfh and B cells in HIV-1 infected individuals may affect the response to HBV vaccine. The general objective of this thesis was to elucidate HBV vaccine response in HIV-1 infected children receiving ART, to assess functional and phenotypic properties of pTfh cells in HBV vaccinated children and to study whether HBV vaccination may have a role in reducing the size of HIV-1 reservoirs. In **paper I**, we showed reduced frequencies of pTfh cells in HIV-1 infected children compared to healthy controls and of pTfh cells expressing the co-stimulatory molecules ICOS and PD1, important to mediate the interaction of Tfh cells with B cells. The frequency of IL-4 expressing pTfh cells and of resting memory B cells was also lower in infected children; on the contrary, an expansion of exhausted memory B cells was detected in this group. In **paper II**, all children who received HBV vaccination, except four, displayed a strong vaccine response at 1 month post-vaccination. Lower plasma levels of anti-HBs antibodies (Abs) were measured in HIV-1 infected children compared to controls at 1 month and 6 months post-vaccination. HIV-1 infected children had elevated plasma CXCL13 levels compared to controls at all time points; changes in plasma CXCL13 concentration were however not observed following vaccination. As the functional and phenotypic properties of pTfh cells were similar in both groups pre- and post-vaccination, alterations in pTfh properties could not explain the reduced vaccine response in HIV-1 infected children. In a yet unpublished study, we showed an altered frequency of B cell subsets in HIV-1 infected children which correlated with anti-HBs Ab titers after vaccination. In **paper III**, the number of HIV-1 DNA copies in PBMCs was unchanged after vaccination with a combined HBV and HAV vaccine in HIV-1 infected children; however, 54% of these individuals showed a decline in the size of HIV-1 DNA reservoir after vaccination. The change was most likely related to vaccination since the children were on ART for a median of 7.2 years and had therefore likely reached a plateau phase for HIV-1 DNA decay after ART initiation. EM CD8+ T cells were the stronger predictors of the change in HIV-1 DNA copies using multivariate analysis. In conclusion, three doses of accelerated HBV vaccination induced high anti-HBs Abs in both HIV-1 infected and control children. A rapid decline of anti-HBs Abs in plasma after 6 months from vaccination suggests the need of an additional booster dose for HBV vaccine. The role of HBV vaccination in reducing HIV-1 DNA reservoirs should be investigated further.

LIST OF SCIENTIFIC PAPERS

I. **Bekele Y**, Amu S, Bobosha K, Lantto R, Nilsson A, Endale B, Gebre M, Aseffa A, Rethi B, Howe R, Chiodi F. Impaired Phenotype and Function of T Follicular Helper Cells in HIV-1-Infected Children Receiving ART. Medicine (Baltimore). 2015 Jul; 94(27):e1125. doi: 10.1097/MD.0000000000001125.

II. **Bekele Y**, Yibeltal D, Bobosha K, Andargie TE, Lemma M, Gebre M, Mekonnen E, Habtewold A, Nilsson A, Aseffa A, Howe R, Chiodi F. T follicular helper cells and antibody response to Hepatitis B virus vaccine in HIV-1 infected children receiving ART. Sci Rep. 2017 Sep 19;7(1):5805. doi: 10.1038/s41598-017-09165-6.

III. **Bekele Y**, Lantto R, Soeria-Atmadja S, Nasi A, Zazzi M, Vicenti I, Naver L, Nilsson A, Chiodi F. HBV vaccination in HIV-1 infected young adults: a tool to reduce the size of HIV-1 reservoirs? Front Immunol. 2018 Jan 10;8:1966. doi: 10.3389/fimmu.2017.01966.

PUBLICATIONS NOT INCLUDED IN THE THESIS

I. Chiodi F, **Bekele Y**, Lantto Graham R, Nasi A. IL-7 and CD4 T Follicular Helper Cells in HIV-1 Infection. Front Immunol. 2017 Apr 20;8:451. eCollection 2017. doi: 10.3389/fimmu.2017.00451.

II. Amu S, Lantto Graham R, **Bekele Y**, Nasi A, Bengtsson C, Rethi B, Sorial S, Meini G, Zazzi M, Hejdeman B, Chiodi F. Dysfunctional phenotypes of CD4+ and CD8+ T cells are comparable in patients initiating ART during early or chronic HIV-1 infection. Medicine (Baltimore). 2016 Jun; 95(23):e3738. doi: 10.1097/MD.0000000000003738.

CONTENTS

LIST OF ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
AM	Activated memory
Anti-HBs	Hepatitis B surface antibody
APC	Antigen presenting cell
ART	Antiretroviral therapy
Ascl2	Achaete-scute homologue 2
BAFF	B cells-activating factor
Bcl-6	B cell lymphoma 6
BCR	B-cell receptor
BR3	BAFF-receptor 3
cccDNA	Covalently closed circular DNA
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CM	Central memory
CRF	Circulating recombinant forms
CXCL	C-X-C chemokine ligand
CXCR	C-X-C chemokine receptor
DAA	Direct-acting antiviral agent
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
EC	Elite controller
ELISA	Enzyme-linked immunosorbent assay
EM	Effector memory
FDCs	Follicular dendritic cells
FOXP3	Foxhead box P3
GC	Germinal center
gp	glycoprotein
HAV	Hepatitis A virus
HBcAg	Hepatitis B core antigen
HBeAg	Hepatitis B e antigen
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HEU	HIV-1 exposed uninfected
HIV	Human immunodeficiency virus
HIV-1	Human immunodeficiency virus type-1
HIV-2	Human immunodeficiency virus type-2
HTLV	Human T-cell lymphotropic virus type
ICOS	Inducible costimulator
ICOSL	Inducible costimulator ligand
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IT	Immature transitional
LPS	Lipopolysaccharide
mcg	Microgram
MHC	Major histocompatibility complex
MTCT	Mother-to-child transmission
MZ	Marginal zone

NK	Natural Killer
PBMC	Peripheral blood mononuclear cell
PD	Programmed cell death
pgRNA	Pre-genomic RNA
PLWH	People living with HIV
PMTCT	Prevention of mother-to-child transmission
RM	Resting memory
RNA	Ribonucleic acid
sCD14	Soluble CD14
SHM	Somatic hypermutation
SIV	Simian immunodeficiency virus
TCR	T cell receptor
TEMRA	Terminally differentiated effector memory
Tfh	T follicular helper
Tfr	T follicular regulatory
TGF	Transforming growth factor
Th	T helper
TLM	Tissue-liked memory
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Treg	T regulatory
VOA	Virus outgrowth assay

1 CHAPTER I: INTRODUCTION

1.1 BACKGROUND

Infection with HIV-1 is a compelling public health problem and among the leading causes of death in sub-Saharan Africa. Globally, the virus infected more than 70 million people and killed about 35 million lives so far. Sub-Saharan Africa is disproportionately affected by HIV-1 burden as infections in this part of the world accounted for two-third of HIV-1 infection worldwide. In 2017, around 20.9 million people living with HIV-1 (PLWH) were accessing antiretroviral therapy (ART) globally. In pregnant women living with HIV-1, ART prevents mother-to-child transmission (PMTCT) of the virus; 76% of HIV-1 infected mothers were accessing the treatment in 2016 [1, 2]. HIV-1 and Hepatitis B virus (HBV) have common modes of transmission and, in co-infected individuals, the clinical outcomes linked to these two infections are exacerbated [3]. HBV infection is vaccine preventable; vaccine specific antibodies, however, wane quickly in HIV-1 infected patients. Thus, both ART treated and untreated HIV-1 infected individuals, even if vaccinated, will be at risk of acquiring HBV infection and related complications [4-7]. Immune response to HBV vaccine improved in HIV-1 infected individuals by increasing the vaccine dosage, the frequency of boosters and the use of different types of adjuvants [8, 9].

1.2 HIV-1 EPIDEMIOLOGY

In 1981, five young homosexual men were treated for *Pneumocystis carinii* pneumonia in Los Angeles hospitals. These patients had a history of cytomegalovirus (CMV) and mucosal candida infections and they displayed a very poor response to mitogens and antigens [10]. Unusual forms of Kaposi's sarcoma were also seen in young homosexual men who were positive for CMV antibodies and hepatitis B surface antigen (HBsAg) or hepatitis B surface antibody (anti-HBs) [11]. Patients were also experiencing severe deficiency in cellular immune responses and this syndrome was later on named acquired immunodeficiency syndrome (AIDS) [12]. The causative agent of AIDS was discovered through repeated isolations of the virus, initially named human T-cell lymphotropic virus type III (HTLV-III), from AIDS and pre-AIDS patients; a serological test was developed soon thereafter for screening blood before transfusion [13-17]. In 1986 the virus was renamed to human immunodeficiency virus (HIV) by the International Committee of the Taxonomy of Viruses (ICTV) [18].

Retroviridae is a large family of viruses which relies on a ribonucleic acid (RNA)-dependent deoxyribonucleic acid (DNA) polymerase (reverse transcriptase) capable of synthesizing DNA from the virus RNA. Of the seven Retroviridae sub-families, delta-retroviruses comprise the T lymphotropic viruses HTLV-1 and HTLV-2 which cause T cell lymphoma and neurological disease, and the lentiviruses HIV-1 and HIV-2 which cause AIDS [19]. HIV-2 was isolated from West Africa patients in Guinea Bissau and Cape Verde [20]. Chimpanzees and/or gorilla are more likely to be the source of the initial HIV-1 transmission to humans; on the other hand, sooty mangabeys are likely the source of HIV-2 since a closely related group of viruses, called simian immunodeficiency viruses (SIVs), was isolated from primates in sub-Saharan Africa [21, 22].

There are four distinct HIV-1 groups; M (major or main), N (non-M or non-O) [23], O (outlier) [24] and P [25, 26]. HIV-1 group M is responsible for the world-wide pandemic and is subdivided into 9 clades or subtypes (A to D, F to H, J and K) and CRFs (circulating recombinant forms) originated through the recombination of subtypes are also included in M [27]. Subtype C is the most dominant (48%) of all subtypes of HIV-1 M group and this subtype is present in India, Southern Africa, and Eastern Africa including Ethiopia. HIV-1 M subtypes A (12%) and B (11%) have a large distribution globally. Subtype B dominates in Western and Central Europe, Australia, North America, the Caribbean and Latin America. Subtype A is present in Western and Eastern Africa, Eastern Europe, Russia and Ukraine [28]. Subtype B (47%) was the dominant subtype in HIV-1 infected individuals in Sweden, followed by subtype C and CRFs; an increase in non-B subtypes over-time was due to immigration from Africa and Thailand [29]. In Ethiopia, the HIV-1 epidemic is predominantly due to the C-subtype, which can be subdivided into sub-cluster C and sub-cluster C' [30, 31]. HIV-2 is a less virulent virus with a lower transmission efficacy; only eight (A-H) groups were identified for HIV-2 [32].

Globally, the incidence of HIV-1 infection decreased from 2.1 million in 2015 to 1.8 million in 2016; the decline in the incidence of HIV-1 infection was estimated to be 11% and 47% since 2010 in adult and children, respectively [1, 33]. The prevalence of HIV-1 increased over the years but the death rate declined through an increased access to ART. A total of 19.5 million people, 54% of 34.5 million adults and 43% of 2.1 million children (Figure 1), were accessing ART in 2016 [1]. Global scale up in the provision and access to ART has led to the removal of HIV from the top 10 causes of death world-wide [34]. The estimated prevalence of HIV-1 in Western and central Europe, and North America was 2.1 million which contributed only to

5.7% of HIV-1 infection globally in 2016. The largest contribution (70%) to HIV-1 prevalence was from Sub-Saharan Africa with an estimated prevalence of 25.5 million infected, 54% of those individuals accessing ART and over 0.7 million deaths reported in 2016 [1]. In Sweden, eleven thousand people are estimated to live with HIV-1 and, among them, less than 100 were children. In 2016, ART coverage was 83% among the total number of people living with HIV-1, and was greater than 95% among children [35]. In Ethiopia, the rate of HIV-1 prevalence in adults is estimated to be 1.1% and 710 thousand people estimated to live with HIV-1, of these 62 thousand were children. The coverage of ART in Ethiopia was 59% in children and adults; however, children ART coverage was very low (35%). The ART coverage among HIV-1 infected Ethiopian pregnant women for PMTCT was 69% with 4200 new HIV-1 infection estimated in 2016 [36].

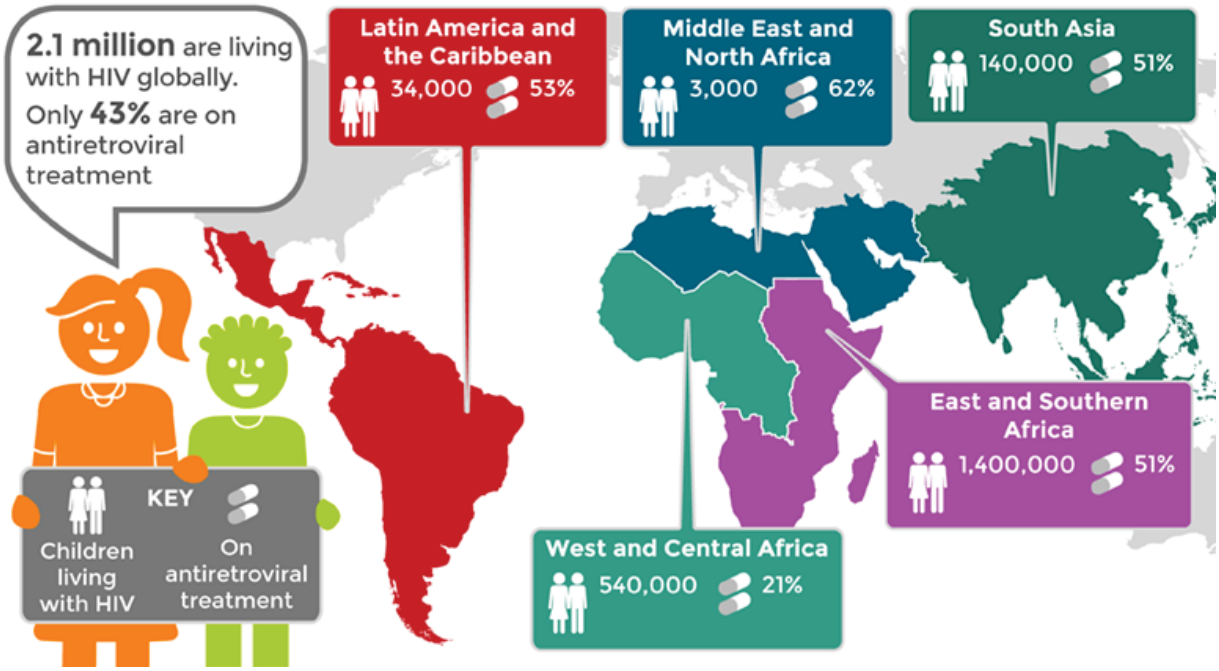

Figure 1. Global epidemiology and access to antiretroviral treatment for HIV infected children in 2016 (Adapted from [37]).

1.3 PATHOGENESIS OF HIV-1 INFECTION

1.3.1 HIV-1 entry and replication

Most of HIV-1 transmissions occur during heterosexual intercourses as the virus passes the mucosal barrier through microlesions in this compartment [19, 38]. Mother-to-child transmission (MTCT) is another route of HIV-1 transmission which may occur at pregnancy,

labour, delivery and breastfeeding. The rate of MTCT is 15 to 45% without any interventions but the transmission can be reduced below 5% through ART administration and other effective interventions during peripartum and postpartum periods [39].

The complex life cycle of HIV-1 is initiated when the viral envelope (env) proteins bind to the virus receptor CD4 on host cells at the site of transmission [40]. The binding of the HIV-1 external envelope glycoprotein (gp120) to the CD4+ receptor on the host T cells triggers a gp120 conformational change thus exposing part of the gp41 protein which will interact with CCR5 and/or CXCR4 co-receptors on CD4+ T cells [41, 42]. Non-syncytium inducting viruses preferentially use CCR5 (R5) as co-receptors in early phase of HIV-1 infection; syncytium inducing viruses which utilize CXCR4 (X4) as co-receptors can be found during the chronic phase in approximately 50% of infected patients [43, 44]. Upon entry into the host cells, the viral genome is reverse transcribed into double stranded DNA (dsDNA). The dsDNA is integrated into the host DNA (provirus stage) where the virus uses the host machinery to produce billions of virus copies [41, 42].

1.3.2 Acute (primary) HIV-1 infection

During the early phase of HIV-1 infection and for the subsequent course of the disease, HIV-1 preferentially infects activated CD4+ T cells expressing CCR5 [45]. The time interval between infection of the first cell and the presence of detectable virus in circulation is called eclipse phase [46]. It is difficult to clearly establish the length of the eclipse phase since, for the majority of HIV-1 infections, the time of exact exposure is unknown; the eclipse phase is however considered to last for approximately 10-14 days. During this phase patients are asymptomatic and there is no reliable laboratory test to detect the presence of HIV-1 [47]. A study conducted in the SIV infected macaque model demonstrated small numbers of SIV RNA copies detected at the site of virus inoculation 3 days after infection; the virus was found in different lymphoid tissues and disseminated to other organs at day 12 post-infection [48]. The next phase of the infection, also known as acute (primary) HIV-1 infection, has been divided into four stages (Fiebig stages I to IV) based on the reactivity of the samples in different assays [46]. During acute infection, patients could be asymptomatic or hospitalized due to severe illness associated with seroconversion [49]; a viral set-point, which can reach up to 10^7 or more HIV-1 RNA copies/ml, can be detected in peripheral blood during acute infection [50, 51]. At this time point, the risk of HIV-1 transmission is higher compared to the chronic phase of infection [38]. The virus disseminates to organs and lymphoid tissues, mainly to the gut-

associated lymphoid tissue (GALT), which is enriched with memory CD4+ T cells [38, 52]. HIV-1 causes severe destruction of the gut memory CD4+ T cells and establishes viral reservoirs [41].

During the acute phase of HIV-1 infection, infants may experience cough due to pneumonia, diarrhea, fever, lymphadenopathy and skin rash [53, 54]. As MTCT can occur at different time points, the course of the disease associated to HIV-1 infection varies depending on the time point of the infection [55]. A meta-analysis study reported that the death rate in ART naïve children within the first 12 months of life was 52% for children infected during peripartum and 26% for children infected at postpartum [56]. Higher risk of morbidity and mortality, with an increased susceptibility to infectious diseases, was reported for HIV-1 exposed uninfected (HEU) infants compared to unexposed infants [57].

1.3.3 Chronic HIV-1 infection

The chronic phase of HIV-1 infection starts after a sharp decline of HIV-1 RNA levels at the end of acute HIV-1 infection [52]. Reduced HIV-1 viral load could be due to a strong cellular immune response in HIV-1 infected individuals [52, 58]. At this stage, HIV-1 infected individuals are asymptomatic and can live for a decade or longer in the absence of ART [59, 60]. HIV-1 viral load remains stable, even below detection limits, in slow progressors, and the CD4+ T cells counts decline slowly before AIDS-related complications begin [52, 58]. Early initiation of ART significantly changes the course the disease; in fact, in ART treated patients, the median life expectancy of 75 years is shortened only by 7 years [61].

Disease progression in children is more rapid than in adults. For instance, a study conducted in Malawi showed that 89% of HIV-1 infected ART naïve children died at the age of 3 years, due to a high burden of infectious diseases and lack of appropriate treatment for these infections [62]. A study conducted in Rwanda showed that the risk of death in children, who are naïve for ART and without prophylaxis for opportunistic infections, was 45% and 62% at the age of 2 and 5 years, respectively [63]. A study conducted in three Sub-Saharan countries from 2004 to 2009 reported a 4.5% death rate for children who were on ART for an average 2.3 years [64]. A 6.6% mortality rate was recorded in the Asia region (Thailand, Malaysia, India, Indonesia and Cambodia) where 77% of the children were on ART; in this study, however, a higher mortality rate was reported during the first months of ART showing the need of early diagnosis and effective treatment [65]. In addition, a follow-up study which was conducted in

vertically HIV-1 infected children in the US from 1993 to 2006 revealed that the median age of death increased from 8.9 in 1994 to 18.2 years in 2006 [66]; a 24.5% death rate was reported in children who were not receiving ART [66].

A mortality rate (16.85 deaths per 1000 child-year) was reported among HIV-1 infected children from the northern part of Ethiopia; in this group the early age mortality was higher due to lack of timely ART initiation [67]. According to a study conducted in Addis Ababa, 10.4% mortality was recorded and the majority of deaths occurred during the first 6 months of ART initiation [68]. Successful and early ART treatment normalized the CD4+ and CD8+ T cells counts, reduced the viral load to below detectable limit, increased the survival of HIV-1 infected individuals and decreased HIV-1 transmission [69, 70]. HIV-1 infected children can reach adulthood age with proper management of opportunistic infections (OIs); however, early initiation of ART and high levels of adherence to treatment is critical [71, 72].

CD4+ T cells count below 200 μl/ml and/or evidence for at least one AIDS-defining conditions are used to diagnose HIV-1 infected patients with AIDS, according to CDC definitions [73]. AIDS-defining conditions include mycobacterium infection at any site, recurrent bacterial infection, HIV-1 related encephalopathy, wasting syndrome associated with HIV-1 and herpes simplex infection [73]. Prior to the introduction of ART in the clinical management of HIV-1 infection, AIDS diagnosis was associated with fatal outcome within a short period. While ART has significantly reduced the burden of AIDS, patients are still suffering from serious non-AIDS morbidity and pill burden [74].

1.3.3.1 HIV-1 Reservoirs

Even in the absence of detectable HIV-1 RNA during ART treatment, integrated HIV-1 proviral DNA persists in tissues [75] and in peripheral blood mononuclear cells (PBMCs) [76]. HIV-1 predominantly persists in resting central memory (CM) and transitional memory (TM) CD4+ T cells due to the fact that these cells can live for an average of 25 years [77-79]. Tissue T follicular helper (Tfh) cells, which reside in the lymph node within the germinal center (GC), are also major HIV-1 reservoirs [80]. Non CD4+ T cells including follicular dendritic cells (FDCs) [81], macrophages (in the lung (alveolar macrophages) [82], liver (Kupffer cells) [83, 84], bone marrow (osteoclasts) [85] and central nervous system (microglial cells) [86]), monocytes [87, 88] and dendritic cells (DCs) [89] are also involved in HIV-1 latency. Thus, any approach to reactivate HIV-1 in latently infected cells should consider all possible virus

reservoirs in the tissues and peripheral blood for successful functional or sterilizing cure of HIV-1 infected individuals [90, 91].

Several studies demonstrated that HIV-1 reservoirs in infected individuals persist even after protracted ART treatment [92-95]. Early ART initiation in vertically HIV-1 infected children induced a size reduction of the virus reservoirs and decay over time [96, 97]. Before initiation of ART, adult HIV-1 infected individuals had a similar size of the virus reservoirs in circulation during acute and chronic HIV-1 infection; the integrated HIV-1 DNA was significantly reduced after 12 months of ART in early treated but not in chronically infected patients [98]. These findings suggest that the timing of ART initiation, but not the length of ART, is the most important factor to reduce the size of virus reservoirs [99].

A study was conducted to assess the timing of virus reservoir seeding by infecting rhesus monkeys with SIV through the intra-rectal route followed by ART initiation at 3, 7, 10 and 14 days from virus inoculation. Rhesus monkeys treated with ART at day 3 from mucosal SIV infection showed undetectable viral RNA, and undetectable humoral and cellular responses to SIV proteins using Enzyme-linked immunosorbent assay (ELISA) and Enzyme-linked immunospot assay (ELISpot), respectively [100]. Integrated SIV DNA was also undetectable in PBMCs; low, detectable levels of proviral DNA were however measured in lymph nodes and gastrointestinal mucosa mononuclear cells, suggesting that SIV seeding may have occurred during the primary infection phase before detection of SIV RNA in the circulation [100].

A child denominated the ‘Mississippi baby’ had been on ART for 18 months immediately after birth with treatment discontinued for the following 27 months; the child had undetectable viral HIV-1 DNA and RNA copies up to 30 months of age suggesting that very early ART initiation may impact on the establishment of latency [101]. Regrettably, an HIV-1 rebound was detected in plasma during a routine follow-up at the age of 46.4 months in the absence of any apparent clinical symptoms of acute HIV-1 syndrome. The viral load dropped, the frequency of CD4+ T cells increased and the CD4/CD8 T cell ratio normalized within 72 hours from ART re-introduction [102]. Another case report showed that in a child who was on ART from 30 min following birth, HIV-1 serological tests were negative and HIV-1 RNA and DNA levels undetectable. In this child, ART treatment was interrupted at 48 months of age but, unlike the ‘Mississippi baby’, the virus rebounded quickly within days from treatment interruption; HIV-1 RNA declined to undetectable limits after ART was re-initiated at 6 weeks from treatment interruption [103]. Among 100 perinatally infected children, only one child treated with ART at

the age of 3 months, with treatment discontinued between 5.8 to 6.8 years of age, showed a stable CD4+ T cell count, very low levels of circulating HIV-1 RNA and DNA for the following 11.5 years [104]. As limited and conflictual results have been shown on elimination/reduction of virus reservoirs upon prolonged treatment, ART interruption should be handled with caution; in addition, an increased risk of immunosuppression after re-initiation of ART has been reported [97, 101, 105].

1.3.3.2 Measuring the size of virus reservoirs

Quantitative virus outgrowth assay (VOA) is the golden-standard method for measuring the size of HIV-1 reservoir in resting CD4+ T cells. The assay measures the production of viral protein or RNA using ELISA or PCR after co-culture of resting CD4+ T cells with irradiated allogeneic PBMCs in the presence of phytohemagglutinin (PHA) [106, 107]. This process is time-consuming, labor intensive, expensive and needs a large volume of blood. Furthermore, VOA only measures the replication-competent virus but not the defective or non-induced proviruses; in fact VOA measured 100 fold less virus reservoirs compared to PCR based assays [108]. PCR based assays have been used to measure all types of proviruses including replication-competent and defective proviruses from PBMCs, total CD4+ or resting CD4+ T cells [109]. Initiation of ART during acute HIV-1 infection may lead to accumulation of defective virus over time which will only be detected using PCR based assays [108]. There is an urgent need of developing a simple, reproducible and accurate assay which measures the true size of virus reservoir from acute and chronic HIV-1 infected individuals (figure 2).

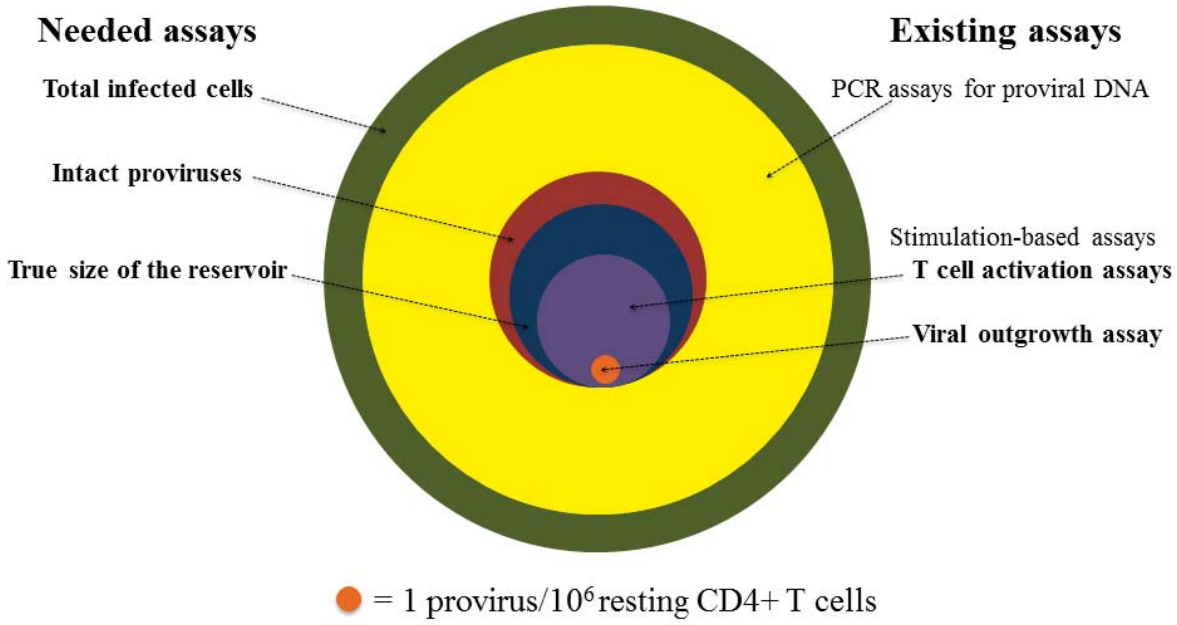

Figure 2. *Vann diagram comparison of proviral HIV-1 DNA measured by different (existing and needed) methods for assessing latent reservoirs (Adapted from [109]).*

1.3.3.3 Microbial translocation and immune activation during HIV-1 infection

Microbial products translocated from the intestinal lumen to the circulation as result of the damage taking place at the epithelial barrier, are associated with immune activation and co-morbidity in HIV-1 infection [110, 111]. Peptidoglycan, lipopolysaccharide (LPS), lipoteichoic acid, flagellin, bacterial DNA/RNA fragments, LPS-binding protein (LBP) and soluble CD14 (sCD14) are some of the biomarkers used to measure microbial translocation in HIV-1 infected and healthy individuals [112, 113]. LBP is an acute phase protein produced by hepatocytes and sCD14 is produced by monocytes in response to LPS. Both LBP and sCD14 are indirect biomarkers produced by innate immune cells during microbial translocation; they bind to- and present LPS [114]. The concentration of LPS significantly increases over the course of HIV-1 infection although the levels of LPS are similar between chronically infected and AIDS patients. Antibiotic treated rhesus macaques showed a marked reduction of LPS levels and gram-negative bacteria in the stool demonstrating the involvement of the gastrointestinal tract in microbial translocation [110].

Several studies pointed out that microbial translocation directly or indirectly increased polyclonal T cell activation through chemokines and cytokines [110, 115, 116]. Chronic HIV-1 infected individuals receiving ART showed a significantly different fecal microbial composition

compared to healthy controls, and plasma levels of LPS positively correlated with plasma immune activation markers including IL-6, TNF-α, C-reactive protein (CRP) and IL-1β [117]. SIV infected macaques treated with sevelamer, a drug which block LPS in the intestine, showed reduced levels of immune activation markers as measured by a lower frequency of Ki67+CD4+ and HLA-DR+CD38+ CD8+ T cells compared to untreated macaques [111]. A study conducted in HIV-1 infected humans confirmed that plasma LPS directly correlated with immune activation markers such as plasma IFN-α and frequency of HLA-DR+CD38+ CD8+ T cells; plasma LPS levels were reduced when the CD4+ T cells count increased upon ART [110]. A study conducted in ART-naïve children also showed that microbial translocation could more often be measured in severely immunocompromised children [115]. Plasma levels of LPS and 16S rDNA were significantly elevated in HIV-1 infected children compared to healthy controls; they were also positively correlated with HIV-1 RNA, expression of immune activation (HLA-DR and CD38) and immune exhaustion (PD1) markers on T cells [115]. Thus, treatment targeting intestinal bacteria may help to reduce polyclonal immune activation and disease progression.

1.3.4 Antiretroviral therapy (ART) for HIV-1 infection

One of the great achievements in the field of HIV-1 research is the design of antiretroviral drugs. The availability and use of ART improves the quality of life of infected individuals, reduces HIV-1 related morbidity and mortality, and changes the natural history of the disease into a chronic manageable condition. Recommendations for timing of ART initiation have been updated at several occasions by WHO, in order to optimally treat HIV-1 infected individuals and to prevent HIV-1 transmission between individuals [118, 119]. Previously the recommendations for ART initiation were based on age, CD4+ T cells count or CD4%, clinical stages, pregnancy status and co-infection(s) of HIV-1 infected individuals [120]. ART treatment interruption was suggested to alleviate pill burden, fatigue, drug toxicity and resistance in HIV-1 infected individuals who had been on ART for a longer time [121, 122]. However, upon treatment interruption, virus rebound took place immediately from long-lived latently infected cells and treatment interruption is not currently considered as an option in the clinical management of HIV-1 infection [123, 124].

The Food and Drug Administration (FDA) has approved more than 25 drugs with six distinct mechanisms of action for the treatment of HIV infection; nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs),

protease inhibitors (PIs), integrase strand transfer inhibitors (INSTIs), CCR5 antagonists and fusion inhibitors [125]. The choice of which type of ART should be administered depends on the patient characteristics (age, weight), drug resistance profiles, availability of the drugs, possible side effects, co-infections and possible drug interactions [126]. Standard ART regimen consists of the combination of at least three drugs to suppress HIV-1 replication and reduce the risk of drug resistance development; the combinations are two NRTIs with one NNRTIs, or PI or INSTI. Clinical evaluations, CD4+ T cells count and HIV-1 viral load are the most common tools to monitor the patient response to ART, drug toxicities, treatment adherence and disease progression. The time interval for monitoring patients on ART depends on the type of drug regimen, the patient age and clinical status; it is however common that clinical monitoring takes place every three to six months [126]. A recent guideline recommended the provision of lifelong ART to all HIV-1 infected individuals regardless of their CD4+ T cell count; this strategy would led to declined levels of viremia in treated individuals and help with the goal of ending AIDS epidemic by 2030 [119].

1.3.5 Adherence to ART

Adherence to ART is a complex behavior which can be influenced by the type of drugs, life style and age of the patients, stage of the disease and the health care system in the country of residence [127]. The adherence rate is low in children compared to adults because children are dependent on their parents or caregivers for monthly checkup and drug refill. Therefore, children adherence will be good only when their caregiver handle the medication properly [127-131]. A meta-analysis study in children and young adolescents showed that in Africa the overall ART adherence was 83.8% varying between 63% in Uganda to 95% in South Africa; the lowest rate (53%) was reported from North America [132]. A study conducted in five health facilities in Rwanda showed that ART adherence was 66% in children less than 15 years of age; HIV-1 positive caregivers facilitated for a higher adherence rate compared to HIV-1 negative [128]. In three regions of Ethiopia, the adherence rate was estimated to be between 84-95% using a caregiver-questionnaire report in the past 7 days prior to interviews [129, 133, 134]. However, a very low adherence was also reported using home-based pill counts which, compared to the caregiver-report, displayed the need for urgent intervention to improve ART adherence in Ethiopia [129].

1.4 HEPATITIS B VIRUS (HBV)

The HBV is an enveloped and partially dsDNA virus classified under the hepadnaviridae family. The virion is composed of an inner nucleocapsid core antigen (HBcAg) and an outer lipid bilayer envelope that contains the surface antigen (HBsAg) [135]. HBV causes a life-treating infection; the long-term complications of HBV infection are severe, including hepatic failure secondary to liver cirrhosis and hepatocellular carcinoma (HCC) [136]. The burden of chronic HBV is due to infection occurring during childhood with clinical complications typically affecting the adult individual [137]. While the HBV vaccine was shown to be highly protective in HIV-1 seronegative individuals, HIV-1 infected children showed less optimal and durable serological responses (Table 1) [138].

1.4.1 HBV epidemiology

The majority of HBV infections occur through perinatal and other transmission routes including sexual contact (common transmission), needles used in common drug-injection, tattooing, unsafe medical practices and contaminated blood or blood products [139]. Around 2 billion people have serological evidence of past or current HBV infection [136]; 257 million people are chronically infected with HBV based on positive HBsAg results and 887 thousand deaths were recorded due to HBV infection related complications in 2015 [140]. According to a WHO report, the prevalence of HBV infection varied markedly throughout different world regions [140]. The virus is highly endemic, with 70–95% serological evidence of past or present HBV infection, in developing regions such as South East Asia, China, sub-Saharan Africa and the Amazon Basin. In these areas, at least 8% of individuals in the population are chronic HBV carriers [141]. HIV-1 and HBV shared similar routes of transmission, thus among the 36.7 million people living globally with HIV-1 infection, 5-10% are co-infected with HBV [142].

Sweden is a low endemic country for HBV infection as the rate of HBsAg positivity was below 1% and less than 5% for overall markers including HBsAg, Hepatitis B core antibody (anti-HBc) and anti-Hbs [143]. In Ethiopia, the prevalence of HBV infection varies across regions; a meta-analysis showed that the pooled prevalence of HBsAg positivity was 7.4% [144]. A study conducted in 57 thousand blood donors from all regions of Ethiopia showed that the overall prevalence of HBV infection was 3.9% in adults; the rate of infection was twice higher in the male compared to female population [145]. HBsAg prevalence was 7% in the capital city, Addis Ababa, as reported from a community-based study [146]. HIV-1/HBV co-infection was 5.5% for outpatient ART treated individuals at the Gondar Hospital [147]. A similar result was

reported from the Mekelle Hospital in northern Ethiopia with 5.9% HBsAg positivity among HIV-1 infected adults receiving ART [148].

Four major HBV serotypes (*adr*, *adw*, *ayr*, *ayw*) and nine sub-serotypes (*ayw*1, *ayw*2, *ayw*3, *ayw*4, *ayr*, *adw*2, *adw*4, *adr*q^+ and *adr*q^-) have been identified based on the HBsAg. These classifications were based on the constant "a" determinant, two mutually exclusive determinants (w/r and d/y) and additional variables. According to the HBV genome sequence, ten genotypes (A-J) have been characterized and further sub-genotypes also introduced. Genotypes and sub-genotypes classifications were based on intergroup nucleotide differences of the complete HBV genome sequence > 8% and 4-8%, respectively [149-151]. Genotypes A and D are more dominant in Africa and Europe; sub-genotype A1 is common in sub-Saharan Africa and A2 in Northern Europe [150, 151]. In Sweden, the genotype D was dominant in patients who visited outpatient clinics, followed by A, C, E and F; the different genotypes illustrated the origin of the patients as genotypes D, A and C were isolated from few Swedish born patients [152]. A study conducted on 391 HBsAg positive samples donated for blood transfusion purpose in five regions of Ethiopia showed that genotype A was predominant, followed by genotype D. The sub-genotype A1 and four D sub-genotypes, D1, D2, D4 and D6, were also reported [153]. HBV serotypes showed that *adw*2 was the leading serotype, followed by *ayw*2 and *ayw*3 [154]. Genotypes are linked with clinical and treatment outcomes: for instance genotype A and B showed a better HBsAg clearance; genotypes C, D and G showed a poor response to IFN-α treatment and genotypes C, D and F had a more significant association with HCC as compared with other genotypes [151].

1.4.2 Pathogenesis of HBV infection

Initial HBV infection could be symptomatic or asymptomatic; most infections are self-limiting in immunocompetent adults, but 5% of these individuals will develop persistent or chronic HBV infection. The proportion of chronic infections varies with the age of infection; 80-90% of infants are infected vertically at birth or horizontally during the first year of their life and 30-50% of children infected before the age of 6 years develop chronic HBV infection [140]. Cellular and humoral immune responses are responsible for successful clearance of HBV infection, although the detailed clearance mechanism is yet not well defined [155]. The virus is non-cytopathic; however, liver injury and related clinical manifestations are triggered by immune-mediated responses of the hosts [156]. HBV infection of hepatocytes is mediated by the interaction of sodium taurocholate co-transporting polypeptide (NTCP) and the pre-S1

region of the HBV surface protein [157]. Upon entry of the virus into the host cell, the partially double stranded (relaxed circular) viral DNA is repaired to form a covalently closed circular DNA (cccDNA) which will be integrated into the hepatocyte genome (Figure 3). Viral cccDNA serves as template to form pre-genomic ribonucleic acid (pgRNA) and all viral transcripts; the virus uses its own reverse transcriptase to form the HBV DNA genome from pgRNA. Another key feature of cccDNA is its ability to persist in the host and to resist current anti-HBV treatments [156, 158, 159].

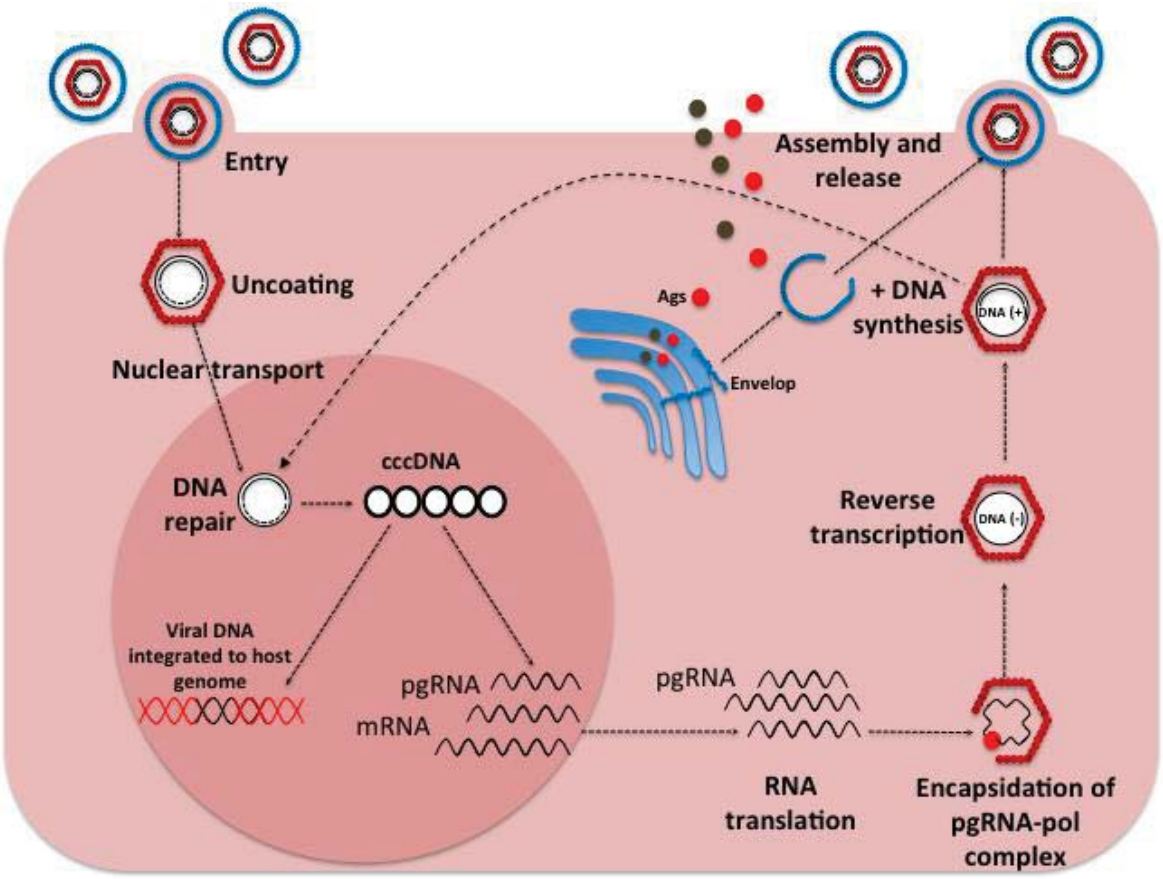

Figure 3. *Schematic representation of the HBV life cycle in human hepatocytes. The steps shown in the figure are: HBV virus entry into an hepatocyte, uncoating of the virus particle, transport of the nucleocapsid to the nucleus, repair of relaxed circular DNA to form cccDNA, integration, transcription, translation, encapsidation, reverse transcription, partially circular + DNA formation, assembly and release [156, 158-160]. cccDNA: covalently closed circular DNA; pgRNA: pre-genomic RNA; Ags: antigens (HBsAg and HBeAg).*

The average incubation time of hepatitis B virus is 75 days but may vary between 30-180 days [140]. The HBsAg and IgM antibodies against the HBV core (anti-HBc IgM) are detected in the

blood of HBV infected individuals [161]. The presence of hepatitis B e antigen (HBeAg) indicates virus replication and infectivity; transition from the presence of HBeAg to anti-HBe seroconversion reflects the transition from active phase to inactive phase of infection [162]. In the chronic phase of infection, 6 months after initial encounter with the virus, the HBsAg can be detected although the HBeAg may not be in circulation [140]. The outcome of HBV chronic infection varies between patients, with the majority being asymptomatic without any liver damage [163]. Co-infections with other viruses [HIV, Hepatitis C virus (HCV), Hepatitis D virus (HDV)], age, family history and alcoholism are some of the risk factors which contribute to the progression of chronic HBV infection to fibrosis, cirrhosis or HCC [164].

Toll-like receptors (TLRs) ensure a first line of defence against viral infection.Upon injection of TLR ligands into mice activation of TLRs takes place with induction of cytokines which inhibit HBV replication; accordingly, activation of TLRs may be a future treatment strategy for chronic HBV infection [165]. Interferon (IFN)-α/β produced by DCs, and IFN-γ released from Natural Killer (NK) cells, NK T cells and Kupffer cells and interleukins (IL-12 and IL-18) are important for viral control without damaging hepatocytes [163, 166]. B and T (CD4+ and CD8+) cells play a pivotal role in the control of HBV infection and determine its outcome as illustrated in Figure 4 [167]. Cytotoxic CD8+ T cells are critical for rapid clearance of virus harbouring cells during acute HBV infection. As a proof of this, HBV DNA was at its pick when chimpanzees were treated with an anti-CD8 antibody; the re-appearance of CD8+ T cells led to a decline in viremia and reduced risk of disease progression [168]. HBV infected individuals who mounted a high HBV-specific CD8+ T cell response during acute infection showed a decline in HBV DNA and HBeAg levels, and liver function assays returned to normal levels [169]. The frequency of HBV-specific CD8+ T cells was reduced after recovery from acute HBV infection; however, the limited number of HBV-specific CD8+ T cells observed in patients at the chronic phase of the disease [170] strengthen the role of CD8+ T cells in viral clearance. On the other hand, CD8+ T cells can cause liver damage in the process of clearing the virus and T cell responses are exhausted in the chronic phase of HBV infection [163]. These exhausted CD8+ T cells have impaired cytokines' production and express multiple inhibitory receptors including programmed cell death-1 (PD1) and cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) [171].

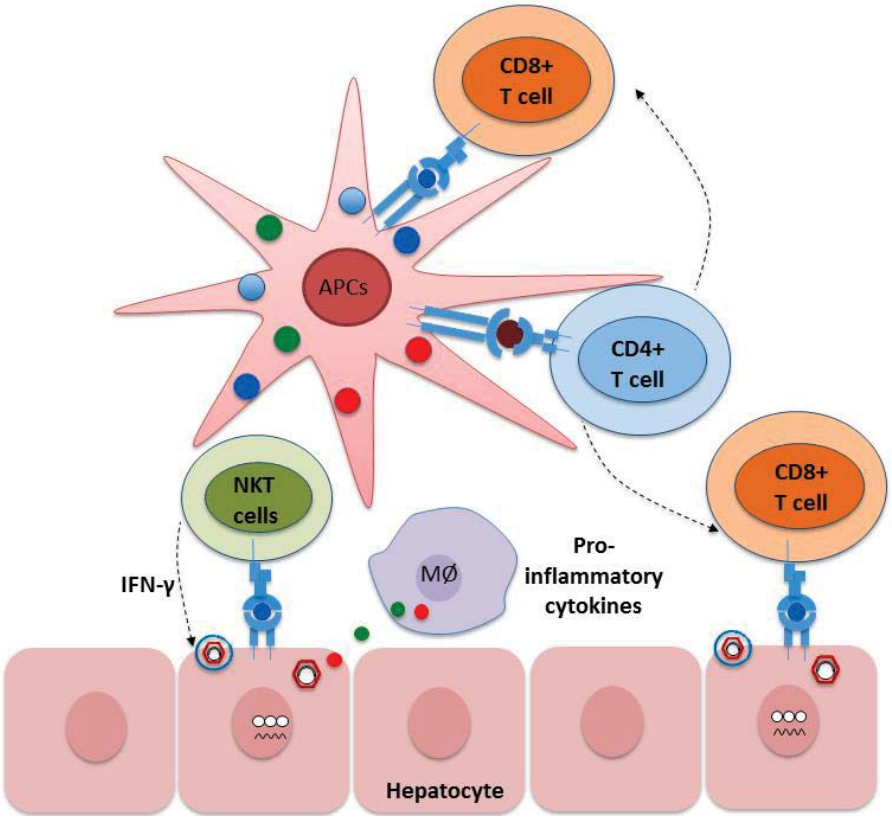

Figure 4. *Schematic presentation of immune responses to HBV infection of human hepatocytes. Components of the innate immunity, including NKT cells and macrophages, recognize infected cells and induce cytokines which will control HBV replication without damaging the hepatocytes. Antigen presenting cells (APCs) capture, process and present HBV peptides in the context of MHC-I to CD8+ T cells or MHC-II to CD4+ T cells. Activated HBV specific CD8+ T cells interact with infected cells and directly kill them through release of perforin and granzymes or antiviral cytokines like IFN-γ and TNF [156, 158-160, 167]. IFN-γ: interferon-gamma; APCs: antigen presenting cells; MØ: macrophage.*

1.4.3 HIV-1 and HBV co-infection

HIV-1 and HBV share the same route of transmission and a higher prevalence of HBsAg or anti-HBc can be detected in co-infected than mono-infected individuals [172]. Both viruses aggravate the negative effects of the infections and are known to cause chronic illness, cancer and death [3]. HBV vaccine response was low in HIV-1 infected individuals and the chance of reverse-seroconversion was also high in these vaccinated, HIV-1 infected people [173]. CD8+ T cells are both responsible for virus clearance and pathogenesis in HBV infection; however, as

impaired CD8+ T cells were reported during HIV-1 infection the likelihood of clearing acute HBV infection is very low in HIV-1 infected people [168, 174]. Co-infected individuals showed elevated levels of HBV DNA and HBeAg, and the risk of developing chronic HBV was six-fold higher as compared to the mono-infected counterpart [3, 172, 173]. The progression rate to cirrhosis and/or HCC is much faster, and liver related mortality is more common, in co-infected individuals [175].

1.4.4 Treatment of HBV infection

The goal of HBV treatment is to reduce morbidity and mortality related to liver diseases by suppressing HBV DNA, improving liver histology and normalizing liver enzymes [176]. A complete cure means eradication of cccDNA from all infected hepatocytes without reactivation when the patient is off-treatment; nevertheless, there is a gap in chronic HBV infection cure strategy due to persistence of cccDNA in the hepatocytes [176, 177]. The most common drugs for the treatment of chronic HBV infection are IFN and direct-acting antiviral agents (DAAs); DAAs are easy to administer and have less side effects compared to IFN [176]. DAAs act on DNA polymerase (reverse transcriptase) which inhibits HBV DNA synthesis. Meanwhile, entecavir and tenofovir are the most efficient nucleos(t)ides analogs (NUCs) for the treatment of chronic HBV infection [178]. IFN induces the release of intracellular enzymes which degrade viral messenger RNA and inhibit protein synthesis; pegylated IFN-α-2a and 2b are common for the treatment of chronic hepatitis B [178, 179]. According to the American association for the study of liver diseases, pegylated IFN-α2a (for treatment of adults) and IFN-α2b (for children), lamivudine, telbivudine, entecavir, adefovir and tenofovir are approved antiviral agents for the treatment of chronic HBV [176]. For HIV-1/HBV co-infection, tenofovir, entecavir, lamivudine and telbivudine should be considered in the treatment regimen [176].

1.5 HEPATITIS B VACCINE

The use of hepatitis B vaccine has been promoted since the 1980s, based upon reliable results from clinical trials. The vaccine has been available since 1982, with 95% efficacy in preventing HBV infection and its chronic consequences, and it is the first vaccine against a major human cancer [180, 181]. The vaccine is an inactivated product; it is recommended to vaccinate all individuals negative for HBV markers, regardless of their HIV-1 status. In 1991, the WHO recommended to integrate HBV vaccination into all national immunization programs and by the end of 2010, 179 countries had introduced HBV vaccine into their routine vaccination

programs. In Africa, the childhood vaccine coverage is about 75% [181] and Ethiopia introduced HBV vaccine in 2007 with an initial coverage rate of 73%. In 2016, the Ethiopian HBV vaccination coverage rate had increased of 23% compared to 2007, based on childhood immunization official report [182].

1.5.1 HBV vaccine composition

The HBV vaccines are composed of highly purified HBsAg, a glycoprotein of the outer HBV envelope. The first licensed vaccine consisted of HBsAg particles purified from the plasma of chronic HBV infected individuals. With the advent of recombinant DNA technology the HBsAg gene was inserted into plasmids. The recombinant HBV vaccine is produced using yeast and mammalian cells; an adjuvant (aluminum phosphate or aluminum hydroxide) is added with thiomersal as preservative. Several yeast species have been used for the expression of plasmids, namely *Saccharomyces cerevisiae, Pichia pastoris* and *Hansenula polymorpha*. Both plasma-derived and recombinant DNA vaccines are safe and elicited protective antibodies after three or four series of vaccine doses. HBV vaccine can be available in a monovalent or in a combination form mainly with hepatitis A or with other antigens as pentavalent vaccine (diphtheria, acellular or whole cell pertussis, tetanus and *Haemophilus influenzae* type b) [183-185].

1.5.2 Schedules and dosage of HBV vaccine

Antibody levels against HBsAg ≥ 10 IU/L after vaccination are considered protective against HBV infection [186, 187]. A standard HBV vaccination schedule comprising three doses of intramuscular or intradermal injection at 0-1-6 months is most common world-wide [188-192]. However, in HBV hyperendemic countries and in high risk individuals such as injection drug users, prisoners, homeless, travelers and immunocompromised people an accelerated HBV vaccination schedule is recommended since it prevents horizontal infection and increases the vaccine compliance and coverage [193]. The other advantage of accelerated schedule is the ability to elicit higher levels of anti-HBs compared to a standard vaccination schedule [194, 195]. The schedules for accelerated HBV vaccination can be 0-1-2 months or super rapid 0-7-21 days; a fourth dose was also recommended at 12 months to slow down the rapid decline of anti-HBs antibodies [194]. However, a similar HBV vaccine response rate was achieved in standard and accelerated HBV vaccinations [196-198].

The recommended dosage for HBV vaccination is 10 mcg (children) and 20 mcg (adults); increasing the dosage might induce better response in HIV-1 infected patients and individuals in hemodialysis. To prevent repeated exposure to HBV, intramuscular administration of 40 mcg HBV vaccine in deltoid muscle increased vaccine response and protection levels in hemodialysis patients [199]. In a study conducted to address HBV vaccine response among non-responders HIV-1 infected patients, a high-dose (40 mcg) revaccination was administered and the response rate was 66.7% [200].

1.5.3 Longevity of HBV vaccine responses

Protection following HBV vaccination is measured using plasma anti-HBs levels which last up to 22 years after completing full vaccination doses [201, 202]. It was shown that the levels of anti-HBs decline over the years; however, the anamnestic anti-HBs antibody response protected the individual from infection upon exposure to HBV during transfusion [203]. Revaccinating individuals who lost protective HBV antibodies after receiving HBV vaccination at birth has proven this concept; upon booster with a single dose of HBV vaccine a rapid increase in the levels of anti-HBs antibody occurred indicating a persistence of a long-term immune memory to HBV. This process (anamnestic response) is defined as at least a 4-fold increase in anti-HBs antibody concentration after 1 month post-challenge [191, 201, 204-209]. Booster doses should be considered for higher risk and immunocompromised individuals.

1.5.4 HBV vaccine response in HIV-1 infected individuals

The rate of HBV vaccine response is variable among HIV-1 infected individuals; however, poor responses, a rapid decline in anti-HBs levels after a few years from vaccination and/or unresponsiveness to the vaccine are the major issues in this group of patients [194]. Numerous factors have been associated with impaired vaccine response in HIV-1 infection; low CD4+ T cells and high HIV-1 viral loads are some of the main reasons [210]. Moreover, HEU infants also show defective humoral and cellular immune responses to infection and childhood vaccinations [211-213]. A study conducted in Latin America and the Caribbean showed that perinatally HIV-1 infected children had very low (21%) anti-HBs levels after four years from HBV vaccination compared to HEU (38%) children. Vaccine response was associated with the timing of ART initiation, maintenance of the memory B cell response, but was not linked with CD4+ T cells count and viral load [214]. A better vaccine response was recorded from HIV-1 infected children with CD4+ T cells count higher than 350 cells/µl and viral load below 40

RNA copies/ml [215]. In Thailand, HIV-1 infected children who had anti-HBs below protective levels were revaccinated at 0, 2 and 6 months; 92% of the HIV-1 infected children responded to the HBV vaccine and the main reasons for this high HBV vaccine response rate were successful treatment with ART for 31 months before vaccination and HIV-1 RNA copies below detection limits at the time of vaccination [216]. In Cameron, HEU and healthy controls had a similar HBV vaccine response rate; the levels of anti-HBs were however significantly different between the groups with 37% of HIV-1 exposed (infected and uninfected) and 61% of healthy controls displaying anti-HBs above 10 IU/L (protective-response) [217]. Moreover, 43% of HIV-1 exposed exhibited low levels (1-9 IU/L) of anti-HBs compared to 21% healthy controls. On the other hand, HIV-1 infected children displayed either an insufficient (56%) or a protective (20%) response [217]. The longevity of anti-HBs in HIV-1 infected children varies with the time of sampling from the last vaccination; at 5.7 years only 24% of children maintained protective anti-HBs [218] and at 9.6 years 1% [219]. Table 1 summarizes the HBV vaccine response among children and adolescents (HEU, HIV-1 infected and healthy controls) in different parts of the world.

Ray and colleagues showed that doubling the HBV vaccine dosage improved the response rate of non-responder HIV-1 infected participants [220]. Another study conducted in HIV-1 infected adults reported a higher HBV vaccine response in double dose recipients as compared to standard dose [221]. A meta-analysis study revealed a different response rate in double dose HBV vaccine recipients with non-responders showing an excellent response to double vaccine dose [9]. Intradermal vaccination in celiac patients elicited higher anti-HBs compared to the intramuscular route [222]. Similarly, HIV-1 infected individuals exhibited a better response to intradermal vaccination compared to standard dose intramuscular injection [223]. In summary, regular monitoring and booster doses may slow down the rapidly declining anti-HBs levels in HIV-1 infected individuals.

Table 1: HBV vaccine response in HIV-1 infected, HIV-1-exposed uninfected and healthy control children from different parts of the world

References	Country	HBV Vaccine response rate	ART status	Age
[214]	Latin America	20.9% HIV-1 infected and 37.8% HEU	60.2%	0-11 months HEU, 5-51 months HIV-1 infected
[224]	Morocco	76% HC and 29% HIV-1 infected	71.4%	10 months to 10 years
[225]	South Africa	61.1% HC and 15.8 HIV-1 infected	All	5 - 15 years
[217]	Cameroon	60.71% HC, 51.72% HEU and 20% HIV-1 infected	All	6-47 months
[226]	India	94% HIV-1 infected (DD)	81.8%	Under 15 years
[6]	Italy	30.8% HIV-1 infected	94.8%	6-28 years
[227]	The Netherlands	97% HIV-1 infected	89%	1-16 years
[215]	Rwanda	71% HIV-1 infected	All	9.3-14.2 years
[228]	Tanzania	59.5% HIV-1 infected	52.8%	1-10 years
[219]	Thailand	1% HIV-1 infected	All	7.1-12.1 years
[229]	Tanzania	74.3% HC and 25.7% HIV-1 infected	None	2-59 months

HC: healthy controls; HEU: HIV-1-exposed uninfected; DD: double dose

1.6 T AND B LYMPHOCYTES IN VACCINE RESPONSES

Most vaccines induce strong, potent and long lasting immune responses which will enable the host to neutralize pathogens and control the pathogenesis of infectious diseases. Protein vaccines, including HBV vaccine, elicit strong T-cell dependent responses after administration into the skin. APCs (DCs, monocytes, macrophages) capture and process the antigens [230, 231]; they migrate from the skin to draining lymph nodes through lymphatic vessels where they can present peptides in the context of the major MHC complex to T cells [230]. For the generation of long-lasting memory to vaccines, migration of T and B cells to the secondary lymphoid tissues and interaction of these cells in the GC is critical. B and T cells interaction in the GC generates memory B lymphocytes, which contribute to the rapid and effective response occurring upon antigen re-exposure, and long lived plasma cells, which ensures the maintenance of protecting levels of vaccine specific antibodies for many years [232, 233].

Naïve T lymphocytes, both with CD4 helper function and CD8 cytotoxic function, express a T cell receptor (TCR) and co-receptors which recognize cognate antigen peptides presented either within the major MHC-II molecule on the surface of APCs for CD4+ T cells or MHC-I on any nucleated cells for CD8+ T cells [234]. T helper (Th) cells can be divided into Th1, Th2, Th17, Th9, Tfh and T regulatory (Treg) based on the expression of the different cytokines which they produce. The differentiation of specific types of T cells are regulated through transcription factors and cytokines present at the immunological synapsis [235, 236]. Each subtype is governed by the following cytokines: Th1 by IL-12, Th2 by IL-4, Th17 by transforming growth factor (TGF)-β, IL-1β, IL-6, IL-21 and IL-23, Th9 by TGF-β + IL-4, Tfh by IL-21, IL-12, IL-23 together with TGF-β and Treg by TGF-β. Specific subsets of T helper cells are known to have master transcriptional regulators: T-box transcription factor (T-bet) for Th1, GATA-binding protein 3 (GATA-3) for Th2, retinoic acid receptor-related orphan receptor-γt (RORγt) for Th17, Interferon-regulatory factor 4 (IRF-4) and PU.1 for Th9, B cell lymphoma 6 (Bcl6) for Tfh and forkhead box P3 (FOXP3) for Treg cells [236-240].

CD4+ T cells expressing C-X-C chemokine receptor type 5 (CXCR5) provide help to B cells in the lymphoid follicles to differentiate into plasma cells and memory B cells [241, 242]. The expression of CXCR5 promotes the migration of cells towards chemokine C-X-C ligand 13 (CXCL13) in the B cell follicles and down-regulates the expression of the C-C chemokine receptor type 7 (CCR7) to facilitate localization in the follicles [241, 242]. Memory CD4+ T

cells, sorted by CXCR5 expression, upon stimulation with anti-CD3 expressed lower levels of IL-10, IFN-γ and IL-4 compared to Treg, Th1 and Th2 cells, respectively. This finding suggested that CXCR5+ cells are a distinct population of T cells; this concept was also strengthened by the observation that CXCR5+ T cells, when co-cultured with B cells, induced a 15 fold increase in IgM production compared to CXCR5- T cells [241]. Tonsillar CXCR5+ T cells are activated cells with effector function; peripheral CXCR5+ T cells, however, are in a resting state and have a weak migration capacity [241, 242]. In 2009, Bcl6, the lineage defining transcription factor for Tfh cells, was discovered as Bcl6 deficient mice showed a defect in Tfh cells development and impaired GC reactions [243-245].

B-lymphocytes are the central element for humoral immunity and are responsible for antibody production in response to pathogens and vaccinations. After development in the bone marrow, mature naïve B-cells re-circulate to secondary lymphoid organs and, in response to antigen activation, they differentiate into memory B cells and plasma cells [232]. Activated B cells migrate to the T-B cells border to receive co-stimulatory signal from CD4+ T cells; the fate of activated B cells can either be to differentiate into antibody-producing cells through an extra-follicular pathway or migrate to GC [246]. Antibodies generated through the extra-follicular pathway are important for rapid protection and the main pathway of antibody production against polysaccharide bacterial capsules or polysaccharide vaccines [247, 248]. GC B cells upregulate Bcl6 which controls GC reaction and regulates survival of B cells [249]. The expression of CXCR4 by GC B cells maintains their localization in the dark zone where their ligand CXCL12 is highly expressed; stromal cells are the main source of CXCL12 [250]. In the final phases of proliferation and somatic hypermutation (SHM) in the dark zone, GC B cells upregulate CXCR5 and migrate from the dark zone into the light zone where there is a higher CXCL13 concentration [250]. In addition to antibody production, B cells are efficient antigen presenters and can stimulate CD4+ T cells. They also produce cytokines such as IL-2, IL-6, IL-10, IFN-γ and TNF-α which play a vital role in controlling allergies, autoimmunity, malignancies and infections [251, 252].

1.6.1 Differentiation and maturation of Tfh cells

Differentiation of Tfh cells is a multi-stage process initiated by the cognate interaction of DCs, antigen presenter, with naïve CD4 T cells in the secondary lymphoid tissues; this process takes place in the T cell zone. A basic helix–loop–helix (bHLH) transcription factor called achaete-scute homologue 2 (Ascl2) is necessary for the initial stage of development of pre-Tfh cells. A

study conducted in mice showed that the expression of Ascl2 leads to upregulation of CXCR5 and down-regulation of CCR7 expression; these events initiate the migration of naïve T cells from the T cell zone to the B cell follicle [253].

Following the initial step of CD4+ T cell commitment into the Tfh cell lineage mediated by Ascl2, the upregulation of the Bcl6 transcription factor is associated with further differentiation of Tfh cells which includes high CXCR5 expression. The cytokines produced by DCs, including IL-12 and IL-23, are a driving force for Bcl6 expression and Tfh cells' development in humans [254]. In vitro experiments showed that stimulation of human CD4+ T cells with anti-CD3 and anti-CD28 promotes CXCR5 and Bcl6 expressions in presence of cytokines. Bcl6 expression is time dependent and a higher Bcl6 induction was found when IL-12 plus TGF-β, or IL-23 plus TGF-β are provided in culture when compared to IL-12 alone. The expression of Bcl6 is further enhanced by the addition of IL-1β and IL-6 also produced by DCs [240]. Immunization of Bcl6 knockout mice with keyhole limpet hemocyanin (KLH) protein did not result in the upregulation of expression of CXCR5 on T cells and GC B cells; however, they expressed elevated levels of Th1 (IFN-γ), Th2 (IL-4) and Th17 (IL-17) cytokines. Likewise, in Bcl6 knockout mice, the expression of genes responsible for Tfh cells development was highly diminished but the Th1, Th2 and Th17 related genes were elevated [244]. T cells of Bcl6 deficient mice differentiate into other lineages and develop spontaneous Th2 inflammation which leads to myocarditis and pulmonary vasculitis; elevated IL-4 and IL-17 expression levels were measured in T cells of these mice [255-257].

Bcl6 gene upregulation represses the expression of transcription factors for other subsets of T helper cells in a DNA binding dependent manner [244]. Binding of Bcl6 to the promoters of transcription factors important for development of Th1 and Th17 cells suppressed the production of IFN-γ and IL-17 cytokines, respectively [245]. Bcl6 is also important to induce Treg cells in the follicles, known as T follicular regulatory (Tfr) cells, which maintain homeostasis of immune cells in the GCs. In the absence of Bcl6, the frequency of Tfr cells is reduced and the localization of cells in the GC affected, whereas the distribution of Th1, Th2 and Th17 was not changed [258]. Thus, Bcl6 expression is vital for Tfr cells function and Tfr cells control inflammation mediated by Th2 cells.

Ascl2, Bcl6 and CXCR5 upregulation is sufficient for the development of pre-Tfh cells and migration of Tfh cells to the T–B cell border for cognate interaction with B cells. However, additional factors are required for Tfh cell survival and to complete their differentiation.

Inducible costimulator (ICOS) is a member of the CD28 family which is expressed on Tfh cells and controls the migration of pre-Tfh cells into the B cell follicle. This effect of ICOS is dependent on the binding of ICOS ligand (ICOSL) expressed on follicular B cells which do not carry antigens. Tfh cells and GC development is impaired in the absence of ICOS ligation with ICOSL. ICOS deficient mice cannot upregulate CXCR5 expression on CD4+ T cells and, accordingly, the migration of these cells into the B cells zone is impaired. ICOS expression is therefore important for the establishment and maintenance of the Tfh cells phenotype; in the absence of ICOS, the Tfh cell phenotype is reverted [259, 260].

1.6.2 B cells maturation

Humoral immune responses can be T cell independent (towards polysaccharide antigens) or T cells dependent (towards protein antigens). Polysaccharide (PS) vaccines induce a poor immune response and lack efficacy compared to protein vaccines [261]. Marginal zone (MZ) B cells and IgM antibodies are critical for T cell independent responses; there is no SHM due to lack of GC formation upon exposure to, or vaccination with, polysaccharide antigens. MZ B cells carry poly-reactive B- cell receptors (BCRs) and they act like innate immunity components against encapsulated bacteria [262, 263]. Because of their intense and rapid response to capsular antigens, MZ B cells are a bridge between early innate and adaptive immunity. MZ B cells generate short-lived and low-affinity plasma cells through the extra-follicular pathway without inducing memory B cells [264, 265]. Conjugation of polysaccharide antigens with protein converts T cell independent (TI) to T cell dependent (TD) responses; the pneumococcal polysaccharide-protein conjugate vaccine (PCV) is the best example in this context [261, 266].

T cells provide help to B cells to generate high-affinity memory B cells and long-lived plasma cells; these events require the formation of the special GC microenvironment [246, 248]. B cells development is initiated in the secondary lymphoid tissue where they recognize unprocessed or processed antigens with their BCRs [267]. Activated B cells express co-stimulatory molecules, upregulate CCR7 and present antigen within the MHC-II for cognate interaction with T helper cells at the T-B cells border; this process helps B cells to undergo proliferation and differentiation into GC B cells [268, 269]. Migration of B cells and interaction with T cells in the GC is a bi-directional activation signals which helps differentiation and affinity maturation of B cells [248, 269].

1.6.3 Germinal center formation

GCs are transient micro-environments of secondary lymphoid tissues where B cells undergo SHM which leads to differentiation into memory B cells and the production of high-affinity antibodies [246]. Germ-free animals are devoid of GCs since this structure is formed only following response to antigens. When mature B cells meet cognate antigens, after infection or immunization, they become activated and upregulate the expression of CXCR5 [270]. GC B cells proliferate in the dark zone of the follicle; proliferation facilitates the mutation of antibody variable region of the antigen binding segments. During this process, the size of the GC increases due to the continuous proliferation of B cells which are now defined as centroblasts. Centroblasts migrate to the light zone to become centrocytes; this step needs interaction of centrocytes with FDCs and Tfh cells. In the sparsely populated light zone, B cells interact with FDCs and Tfh cells. Centrocytes with higher affinity receive survival signals; cells with low affinity migrate back to the dark zone for further SHM where they rearrange their antigen binding sites or die by apoptosis. Centrocytes differentiate into long-lived plasma cells or memory B cells which ensure a rapid antibody response during secondary antigen exposure [246, 248].

The interaction between Tfh and B cells is governed by costimulatory molecules and cytokines (Figure 5); these include ICOS−ICOSL, CD28−B7, CD40L−CD40, Signaling Lymphocytic activation molecule (SLAM)-associated protein (SAP)−CD84, PD1−PDL1, IL-21−IL-21R and IL-4−IL-4R [271-276]. Impaired GC reaction and formation were observed in the absence of these signaling pathways. ICOS and CD28 are related co-stimulatory molecules which modulate Tfh cells proliferation and differentiation [272, 274, 275, 277]. Patients with ICOS deficiency present with a severely reduced frequency of peripheral (p)Tfh cells suggesting a critical role for ICOS in differentiation of Tfh cells [278]. CD28 is important for differentiation and maintenance of Tfh cells as shown by the finding that mice lacking CD28 expression show a limited Tfh cells differentiation and present with a reduced ICOS expression [279]. CD40L (CD154) is necessary for the maintenance of GC and memory B cells generation [272]; patients with deficiency in CD40L expression showed lack of GC formation and a reduced frequency of pTfh cells, as also found in ICOS deficient patients [278]. SAP knockout mice had profound defects in GC formation and maintenance of Tfh cell differentiation; defective IL-4 and IL-21 was also observed in these mice [280, 281]. PD1 is an inhibitory molecule which is elevated during viral infection; absence of PD1 expression leads to the defective generation of long-lived

plasma cells and reduced *IL4* and *IL21* expressing genes [282]. Tfh cells produce IL-4 and IL-21 and regulate GC responses [283].

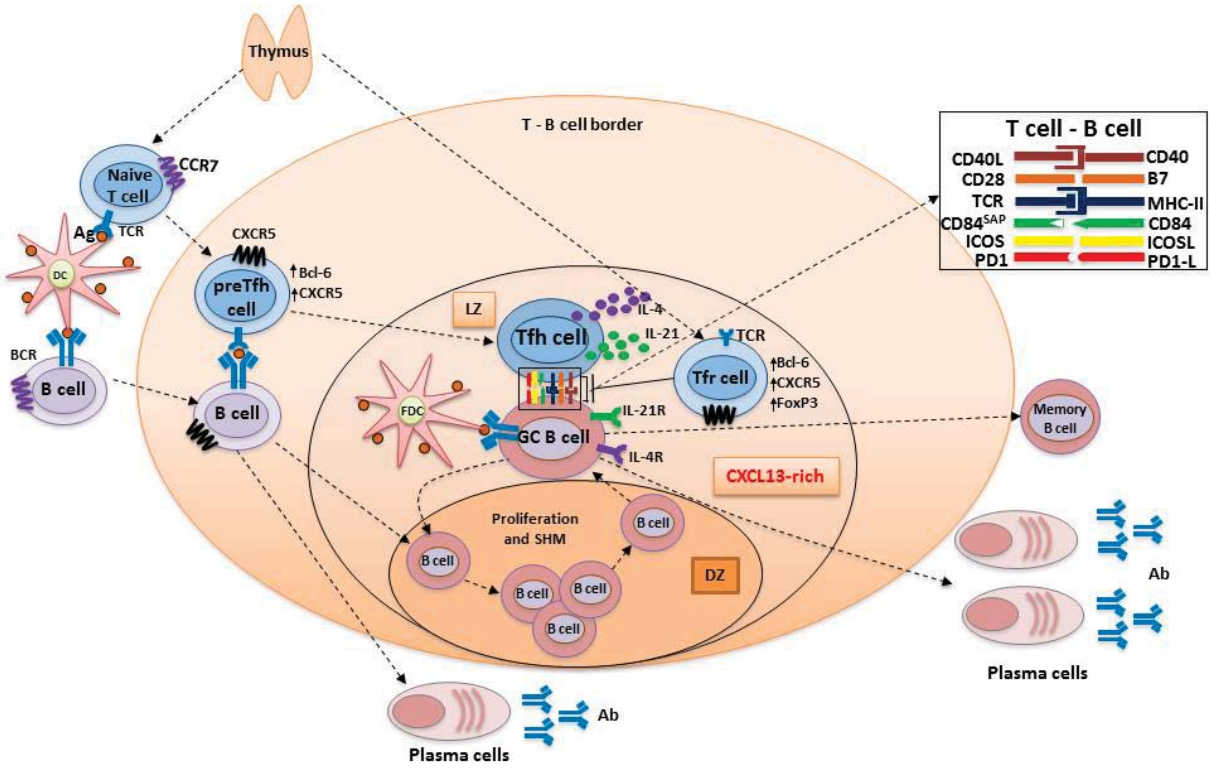

Figure 5*. Germinal center (GC) reaction and GC B cell differentiation to memory B and plasma cells. B cells can differentiate to plasma cells extra-follicularly, or migrate to the dark zone of the GC where they proliferate and undergo somatic hypermutation (SHM) which rearranges the variable regions of the antigen binding sites. Thereafter B cells migrate to the light zone of the follicle where they interact with Tfh cells and FDCs to receive survival signals and differentiate into memory B cells and antibody-producing cells. Tfr cells are important to control Tfh cells and GC formation; they also control the development of autoimmunity [247, 273, 284, 285]. Ab: antibody; Ag: antigen; DZ: dark zone; LZ: light zone; TCR: T cell receptor; BCR: B cell receptor; DC: Dendritic cell; FDC: follicular dendritic cell; Tfh cells: T follicular helper cell; Tfr cell: T follicular regulatory cell; GC B cell: germinal center B cell; IL: interleukin.*

The GC reaction is controlled by special cells called Tfr cells, subset of regulatory T cells [286]. Tfr cells (CD4+CXCR5^{high}PD1^{high}Foxp3+) share the characteristics of both Tfh and Treg cells. Tfr cells express both Blimp-1 and Bcl6; Blimp-1 is known to down regulate transcription factors of Tfh cells [287]. Tfr cells differentiate and proliferate in response to immunization or

infection to regulate the size of GC and the magnitude of antibody response [287, 288]. Mice deficient in Tfr cells showed a higher magnitude of GC reaction and increased antibody production; adoptive transfer of Tfr cells limited GC reactions and the serum antibody levels [288, 289]. A study conducted in mice elucidated [290] that Tfr cells regulated Tfh cells through PD1/PDL1 signaling; PD1 deficient mice had a higher frequency of Tfr cells. *In vitro* co-culture of B and Tfh cells in presence of Tfr cells showed a significant suppression of IgG production due to attenuation of responder cells [290].

1.6.4 The role of Tfh and B cells in vaccine response

Vaccines are the best measure to protect people from communicable diseases. Childhood vaccines provide protection by eliciting antibodies which can neutralize pathogens. Current available vaccines save billions of life; however, we still need to improve the existing vaccines and search for new vaccines for deadly pathogens including HIV-1, Hepatitis C virus, Zika virus, *Plasmodium falciparum* and *Mycobacterium tuberculosis*. The response to vaccines varies with the immunological status, gender, age and genetic background of the individual, in addition to underlying diseases and type and manufacturing of vaccines [291].

Improving GCs reaction will help GC B cells to undergo extensive proliferation and increase the antibody diversity through SHM. A study done to assess the role of IL-7 treatment in influenza vaccine response found that the frequency of vaccine specific Tfh cells was enhanced upon co-delivery of the vaccine with IL-7 fused to the mouse Fc portion (IL-7-mFc); there was no effect of this compound on other subsets of T cells. The enhanced frequency of Tfh cells increased the frequency of GC B cells, as compared with vaccine delivery alone. Blocking IL-7 alone, or in combination with Tfh differentiation cytokines IL-6 and IL-21, significantly reduced the frequency of Tfh cells. In addition, mice vaccinated after CD4+ T cells depletion were unable to generate IgG responses in presence of IL-7, illustrating the crucial role of CD4+ T cells for differentiation of Tfh cells, GC formation and for eliciting high IgG production [292]. A study conducted to assess the adjuvant effect of IFN-α noted the capacity of IFN-α to induce Tfh cells expansion and enhance GCs formation [293].

Several studies showed that memory Tfh cells found in blood are representative of the Tfh cells found in lymphoid tissue [294, 295]; thus studying pTfh cells represents a possible approach to understand the immunology of tissue Tfh cells. The frequency of pTfh cells is associated with vaccine response and, interestingly, an increase in ICOS+PD1+CXCR3+ Tfh cells correlated

with antibody avidity after vaccination with influenza trivalent inactivated vaccine [296]. Influenza vaccines significantly increased the frequency of circulating plasmablasts (CD19+CD20-CD38+ cells) and high antibody levels persisted for 6 months; interestingly, the frequency of IL-21+ICOS+ Tfh cells correlated with hemagglutination inhibition (HAI) titers [297]. Another study on influenza vaccination also indicated that the frequency of ICOS+CXCR3+ Tfh cells correlated with influenza specific antibody responses [298]. A clinical trial for a new vaccine to control Ebola virus outbreak analyzed the immune responses to a recombinant vaccine candidate (rVSV-ZEBOV); it was shown that the frequency of Tfh cells increased after 28 days of vaccination and Th17-like Tfh cells significantly increased after vaccination compared to Th1- or Th2-like Tfh cells [299].

The plasma CXCL13 reflects GC activities and correlates with generation of specific antibodies [300]. This finding could be of help to monitor vaccine responses in lymphoid tissue of humans which are otherwise inaccessible for large clinical studies. However, higher CXCL13 plasma levels are reported in various pathological conditions of humans including immune thrombocytopenia [301], Systemic lupus erythematosus (SLE) [302], rheumatoid arthritis [303], idiopathic pulmonary fibrosis [304] and HIV-1 infection [305, 306]. Therefore, antibody titers to vaccine currently remain the best validated correlate of protective immunity after vaccination [307]. As alluded to above, Tfh cells are important for vaccine response by enhancing GC formation and production of high affinity antibodies.

1.7 VACCINE RESPONSES AND TFH CELLS IN HIV-1 INFECTION

Responses to immunizations are complex, requiring communication between several immune cells which develop during the first year of life [308, 309], a process which may be quantitatively and qualitatively affected by HIV-1 infection. Since HIV-1 infection is primarily a disease of lymphoid tissues and causes massive destruction of CD4+ T cells, these pathological features may not be completely restored by ART [310]. Tfh cells expressed higher levels of PD1 in lymph node tissue from HIV-1 infected individuals; elevated cellular PD1 levels inhibited ICOS expression and the secretion of IL-4 and IL-21 cytokines [311].

The frequency of blood memory B cells was significantly reduced in HIV-1 infected subjects, in both ART-naïve and treated individuals. The decline of memory B cells correlated to reduction of antibody titers against measles, tetanus and pneumococcus in HIV-1 infected patients compared with healthy controls [312-314]. Timing of ART initiation is the major factor

predicting the longevity of B-cell responses to routine childhood vaccines in HIV-1 infected children [315]. The results from the latter study showed that initiation of ART in HIV-1 infected children within the first year of life permits the normal development and maintenance of memory B-cells. On the contrary, the frequency of memory B-cells and the level of antibodies to a specific vaccine were remarkably reduced in children treated later with ART [315]. Experiments conducted in macaques infected with SIV have however indicated that depletion of memory B cells from the blood may be due to an increase in homing of these cells to lymphoid tissues during the acute phase of infection followed by apoptosis in lymphoid tissues, the latter process mediated by death receptors [316]. The presence of HIV-1 infection from birth may completely redirect the immunological process aimed at generating high affinity antibodies and serological memory.

In spite of ART, HIV-1 persists in cellular reservoirs which are mostly represented by resting central memory T cells [317]; it has yet not been studied whether the size of the HIV-1 reservoir has an influence on the response to childhood vaccines in HIV-1 infected children. Other pathological mechanisms taking place during HIV-1 infection, including abnormal immune activation which persists to some level in spite of ART, may affect response to vaccines in children and adults infected with HIV-1.

1.7.1 Impaired B cell distribution and function during HIV-1 infection

B cells play a crucial role in mounting humoral immunity during infections or vaccinations. They can also efficiently present antigens in the context of MHC-II to CD4+T cells and direct the CD4+ T cells response towards Th1/Tfh cells [318]. Functional impairment and alteration in the distribution of B cells have been reported during HIV-1 infection [319-322]. Even though there is no evidence of direct HIV-1 infection of B cells, the virus can bind to mature B cells through complement receptor 2 (CR2) or CD21; this interaction may promote B cell activation by the virus and help virus transfer to T cells while B cells travel to the tissue [323]. The defects in the frequency and quality of B cells in HIV-1 infection vary with the treatment status, but are more pronounced in treatment naïve compared to ART treated patients [321, 324, 325]. A higher frequency of immature transitional B cells in circulation was described in HIV-1 infected children and adults; these cells have a weak proliferation capacity upon stimulation with anti-IgM [326-328]. An increase in tissue like- and activated memory B cells, accompanied by a decline in naïve and resting memory B cells, are other immunological features of HIV-1 infected individuals, including perinatally infected children [329]. A reduced frequency of

resting memory B cells, regardless of viral load titer, was reported in HIV-1 infected Kenyan children; low proportion of pTfh cells were associated with a low proportion of resting memory B cells [330].

Plasma levels of B cells-activating factor receptor (BAFF), an important molecule for B cell maturation and survival, were elevated during acute HIV-1 infection [331, 332] and throughout the course of HIV-1 infection [333]. Higher plasma levels of BAFF were also reported in HIV-1 infected children [334]. However, the expression of BAFF-receptor (R) on B cells was reduced in viremic HIV-1 infected individuals thus likely leading to insufficient survival signals delivered to B cells [335]. Muema and colleagues reported an increase frequency of B cell subsets that expressed low levels of BR3 (also called BAFF-R); elevated plasma BAFF levels were associated with BR3 internalization thus probably directly involved in down-regulation of BR3 expression [334].

In experiments performed to assess the production of immunoglobulins by co-culturing sorted pTfh cells and naïve B cells in the presence of staphylococcal enterotoxin B (SEB), the production of IgM and IgG was lower in HIV-1 infected patients compared to healthy controls [336]. When sorted Tfh cells from lymph nodes were co-cultured with autologous GC B cells in the presence of super-antigen, HIV-1 infected cells produced significantly reduced levels of IgG compared to cells from controls [311]. Similar results were observed in SIV infected macaques when sorted Tfh cells were co-cultured with B cells [311]. Fine needle biopsies of lymph nodes revealed that the frequency of GC B cells and plasma cells were significantly higher in ART naïve compared to healthy controls [337]. Elevated numbers of plasma cells correlated with serum immunoglobulin and contributed to non-specific immune activation [338]. Alterations of memory B cell subsets in the lymph nodes were normalized after 72 weeks of ART and the aberrant expansion of plasma cells and GC B cells were reduced after treatment [339].

1.7.2 Tfh and Tfr cells in HIV-1 infection

Peripheral Tfh cells are affected in frequency and function during HIV-1 infection and this impairment is more pronounced in viremic individuals; patients who were successfully treated showed similar frequency of pTfh cells as healthy controls. A study conducted in children showed that the frequency of pTfh cells (CD4+CD45RO+CXCR5+) was significantly reduced in HIV-1 infected individuals compared to healthy controls [329]. The decline in pTfh cells was not associated with CD4+ T cells count, viral load, timing of ART initiation and clinical

variables. In addition, there was no difference in the frequency of ICOS+ Tfh cells between HIV-1 infected and healthy controls [329]. A study conducted with samples collected during seasonal flu vaccination showed that the proportion of pTfh (CD4+CD45RO+CCR7+CXCR5+) cells was similar in HIV-1 infected children and age matched healthy controls [340]. The genes ABCB1, DUSP4, BST2, SAMHD1 and *IL21* induced from CD4+ T cells are important for pTfh cells proliferations and strongly associated with the production of influenza specific, high affinity antibodies in the responder group. However, the study also suggested that CD4+ T cells induced genes (FAS, CCR5, LAG3 and *IL2*) responsible for inhibition of pTfh cells´ differentiation prior to influenza vaccination in the non-responder group [340]. A lower proportion of pTfh cells was found in ART naïve HIV-1 infected individuals compared to healthy controls; the frequency of Tfh normalized after 48 weeks of ART treatment [336].

Tfh cells and their expression of co-stimulatory molecules were similar between healthy controls, chronic aviremic and elite controllers (ECs). However, aberrant antibody production was observed in chronic aviremic individuals compared to HC and ECs after Tfh cells co-culture with B cells, suggesting a defective T-B cells interaction [341]. Co-culture of pTfh with autologous B cells induced higher IL-2 production in chronic aviremic compared to ECs and healthy controls. IL-2 signaling was reversed by addition of αIL-2 antibody in the co-culture which inhibited Tbet and Blimp-1 expression in Tfh cells, thus significantly increasing the expression of CXCR5 and IgG production [341]. The frequency of pTfh cells (CD4+CXCR5+) was similar between ART treated HIV-1 infected adults and healthy controls [342]. After H1N1 influenza vaccination, co-culture of sorted pTfh cells with autologous B cells supported IgG production in HC and HIV-1 infected vaccine responders but not in vaccine non-responders [342].

Studies showed that abnormal expansion of Tfh cells in the secondary lymphoid organs affected the distribution and proliferation of B cells and resulted in defective antibody production. The frequency of lymph node Tfh (CD4+CXCR5+PD1+) cells were significantly higher in HIV-1 infected compared to uninfected controls; this finding was associated with higher expression of Bcl6 [343]. HIV-1 specific Tfh cells expressed higher levels of IL-21 in chronic infected individuals compared to treated patients [343]. The proportion of splenic Tfh cells was elevated in HIV-1 infected individuals and gene expression of signal transducer and activator of transcription (STAT)-3, important for full differentiation of Tfh, was reduced in the spleen of HIV-1 infected patients compared to healthy controls [344].

A study conducted by Colineau and colleagues demonstrated a significantly higher proportion of splenic Tfr cells in HIV-1 infected patients and linked the alteration of B cell responses with a depressed production of IL-4 and IL-10 by Tfh cells which are necessary for B cells maturation [344]. HIV-1 infected individuals had the highest absolute numbers of Tfr cells compared to uninfected controls in all section of the lymph nodes, which affected the GC reaction and humoral immune responses [345]. A marked increase of Tfh and Tfr cells was observed in lymph nodes of chronically SIV infected rhesus macaques compared to acutely infected and non-infected animals [346]. The ratio of Tfh cells to Tfr cells was low in chronic SIV infection and the frequency of Tfr cells out of Tfh cells was also reduced in SIV infected compared to uninfected macaques; this phenomenon may contribute to the increase in the frequency of Tfh cells in the chronic phase of the infection [346]. However, a lower frequency of Tfr cells was reported in chronic SIV infected macaques and associated with expansion of Tfh cell in the lymph nodes [347]. Taking the above points together, defective phenotypes and functions of Tfh and Tfr cells during chronic HIV-1 infection were associated with impaired B cell frequency and function.

1.7.3 Tfh cells as HIV-1 reservoirs

A complete eradication of HIV-1 from the circulation and tissue is still impossible due to the persistence of the virus in tissue reservoirs [348, 349]. ART has a low penetration capacity into the GCs, as a study showed that intracellular concentrations of ART drugs were low in lymph nodes compared to peripheral blood [350]. Moreover, there was a direct correlation between the amount of virus bound to FDC and the number of HIV-1 RNA positive CD4+ T cells in the tissue, although peripheral blood HIV-1 RNA copies was below the detection limits; these findings suggest that there is an ongoing viral replication and production in the tissues during ART [350]. Pallikkuth and colleagues performed *in vitro* activation of sorted pTfh ($CCR7^{+}CXCR5^{+}$) or non-pTfh ($CCR7^{+}CXCR5^{-}$) cells from healthy donors with phytohemagglutinin (PHA) in the presence of green fluorescent protein (GFP) expressing HIV-1; pTfh cells were more susceptible to HIV-1 infection compared to non-pTfh cells [80]. In cells extracted from the tonsils, GC Tfh cells were more permissive than extra-follicular cells for *ex vivo* infection with GFP expressing HIV-1. Moreover, GC area of the follicles had the highest frequency of HIV-1 RNA positive cells compared with the non-GC area in lymph nodes collected from ART naïve HIV-1 infected patients without AIDS [351]. Total HIV-1 DNA levels were unchanged in pTfh cells after 48 weeks of ART suggesting that pTfh play an

important role as virus reservoirs [80]. CCR5-using virus preferentially infected human and macaques Tfh cells [352, 353]. In humanized mice, CXCR5+PD1+ Tfh cells exhibited a high permissivity to HIV-1 infection and these cells expressed high levels of CCR5 and CXCR3 [354]. Overexpression of Bcl6 increased the susceptibility of Tfh cells to HIV-1 infection and significantly facilitated HIV-1 replication; Bcl6 may thus represent a future target to reduce virus reservoirs in the tissues [355].

The size of virus reservoirs varies with timing of ART initiation; a study conducted by Amu *et al* reported a significantly higher number of HIV-1 DNA copies in PBMCs of late treated compared to early treated HIV-1 infected individuals [99]. When the size of virus reservoirs was compared between ECs and ART treated HIV-1 infected individuals, a significantly elevated number of HIV-1 DNA copies was detected in pTfh cells of ART treated compared to ECs suggesting that some unknown mechanism may account for virus clearance in ECs [356]. Ultrasound-guided fine needle biopsies collected from inguinal lymph nodes showed that frequency of Tfh (PD1+CXCR5+) out of memory CD4+ T cells was higher in ART naïve patients compared to healthy controls. The study also confirmed that HIV-1 DNA copies were elevated in lymph nodes compared to peripheral blood; this difference was only observed in ART naïve but not in treated patients [337]. Samples collected from lymph nodes showed that ART naïve individuals had an elevated frequency of Tfh cells compared to healthy controls and patients treated with ART for 72 weeks [339]. This study also revealed that Tfh cells (CXCR5+PD1+) carried a higher number of HIV-1 DNA copies than CXCR5+PD1- cells and that the size of the virus reservoirs was reduced after ART initiation [339]. The expansion of Tfh cells correlated significantly with plasma viral load, suggesting a role of Tfh cells in virus replication and production [339]. *Ex vivo* experiments by Miller and colleagues showed that Tfr cells were highly permissive for HIV-1 infection compared to Tfh cells when tonsil cells from HIV-1 uninfected children were cultured with CCR5-tropic HIV-1 isolates [357]. Thus, early initiation of ART reduces the size of virus reservoirs and maintains the integrity of Tfh cells in the tissue of HIV-1 infected individuals, which in turn helps GC formation and development GC for better immune responses to vaccines.

2 CHAPTER II: OBJECTIVES

The aims of this thesis are:

- to evaluate the functional and phenotypic properties of Tfh cells in ART treated HIV-1 infected and healthy control children **(paper I)**
- to measure serological and Tfh cell responses to HBV vaccine in HIV-1 infected children receiving ART and healthy children with no prior exposure to HBV vaccine or infection **(paper II)**
- to assess the role of hepatitis B vaccination in reducing the size of HIV-1 reservoirs in ART treated children **(paper III)**
- to investigate the possible impairment of B cell subsets of HIV-1 infected children in relation to HBV vaccine responses **(preliminary results)**

3 CHAPTER III: MATERIALS AND METHODS

All studies were performed with specimens obtained from HIV-1 infected and healthy control children from Addis Ababa, Ethiopia and Stockholm, Sweden. The experiments were approved by the AHRI/ALERT Ethical review committee, National Research Ethics Review committee of Ethiopia and the Ethical committee of Karolinska Institutet. The study purpose, benefit and possible discomforts were clearly explained to the parents and then written informed consent was collected. Blood samples were collected by experienced nurses, to minimize the discomfort and pain during sample collection.

Study participants and cohorts

Study I

A cross sectional study was conducted between 2013 and 2014; 48 HIV-1 infected and 55 healthy controls were recruited from All Africa Leprosy, Tuberculosis and Rehabilitation Training (ALERT) Center of pediatric ward and Woreda 02/03 clinic, Addis Ababa, Ethiopia. Blood samples were collected from children between 2–7 years of age. All HIV-1 infected children were on ART and only for 12 children (26.1%) treatment was initiated before 12 months of age; children received ART for an average period of 37.0 months (range 8–66 months). Twenty children had HIV-1 RNA copies between 200 and 582000 copies/mL and eight of them had a viral load higher than 10000 copies/mL.

Study II

This was a prospective cohort study conducted at the Zewditu Memorial Hospital and the Pediatric department of ALERT Center in Addis Ababa, Ethiopia. Age matched healthy controls were recruited from two childcare centers in Addis Ababa (Muday Charity organization and SOS village). HBV vaccine was administrated intramuscularly to a total of 63 healthy controls and 49 HIV-1 infected children. All HIV-1 infected children were on ART for a mean period of 41.8 months. Prior to HBV vaccination, 73.5% (36) of the HIV-1 infected children had plasma HIV-1 RNA copies below 150 copies/ml and only 18.4% (9) of them had over 1000 copies/ml.

Study III

This was a prospective study conducted at Karolinska University Hospital enrolling 22 HIV-1 infected children (8 males and 14 females) for HAV and HBV vaccination with Twinrix (GlaxoSmithKline AB). The median age was 15 years (range 6–18 years). The viral load at the time of vaccination was undetectable (<20 copies/mL) and the median CD4+ T cells count was 715 cells/μL. All children were on ART and for median length of 7.2 years.

Preliminary results

A cross-sectional study was conducted in 54 healthy controls and 53 HIV-1 infected children who received hepatitis B vaccination in Ethiopia. The mean age of the healthy controls and HIV-1 infected children was similar. The mean CD4+T cell count of HIV-1 infected children at the time of sample collection was 942 cells/μl. Fifteen of the HIV-1 infected children were naïve for ART and 38 received ART for a mean of 44.6 months.

Hepatitis vaccination of children (Papers II and III)

Recombinant DNA (rDNA) Hepatitis B vaccine, which consists of purified HBsAg was administered to Ethiopian children **(paper II and preliminary results)**. For the Swedish cohort, Twinrix, a combination of vaccines against HAV (inactivated) and HBV (recombinant), was administrated intramuscularly **(paper III)**. All children received three doses (each of 10 mcg) of vaccine with 4-weeks interval (accelerated vaccination schedule) between the doses. There was no severe adverse event reported upon vaccination in both study cohorts except that children felt pain at the site of injection.

Peripheral blood mononuclear cells (PBMCs) isolation (all papers)

Blood samples were collected at baseline (prior to vaccination) and at 1 and 6 months from the last dose of vaccine (**papers II, preliminary results** and **paper III**); only one sample was collected at one time point for **paper I**. After the plasma fraction was collected from the samples, whole blood was diluted with *RPMI-1640* and layered in *Falcon*™ 15 mL Polypropylene conical *Tubes* which were filled with 3 ml Ficoll-Paque. PBMCs were isolated by standard Ficoll-Paque density gradient centrifugation. PBMCs were then cryopreserved in liquid nitrogen (-160°C) until needed. In **paper I**, additional 400 μl of whole blood were

transferred to standard flow cytometry staining tubes and lysed with red blood cells lysing solution prior to B cell immunostainings.

Plasma HIV-1 viral load determination (All papers and preliminary results)

RNA was extracted from plasma and HIV-1 RNA levels were measured using Quantitative Real-Time HIV-1 assay.

Measurement of total PBMC HIV-1 DNA (paper III)

DNA was extracted from PBMC manually with High Pure Viral Nucleic Acid Kit. Light Cycler 96 system (Roche) was used to measure total HIV-1 DNA copies in PBMCs.

Determination of plasma anti-HBs, anti-HAV and CXCL13 titers (paper II, III and preliminary result)

Anti-HBs Monolisa Plus assay **(paper II, preliminary results and paper III)** and Human CXCL13 DuoSet ELISA **(paper II**) were used to measure the plasma levels of anti-HBs and CXCL13 chemokine, respectively. Total (IgG and IgM) HAV antibodies, and IgM on a separate test, were measured by electro-chemiluminescence immunoassay **(paper III)**. The assays were run according to the instruction provided by the manufacturers and all samples and standards were tested in duplicate.

PBMCs stimulation (paper I and II)

PBMCs were stimulated with PMA (phorbol 12-myristate-13-acetate) and Ionomycin in the presence of Golgistop **(paper I)**. In **paper II,** PBMCs were cultured with BD FastImmune CD28/CD49d costimulatory complex alone or together with the HBsAg protein. At day 5, Golgistop was added to all culture conditions to block release of cytokines from cells.

Flow cytometry (all papers and preliminary results)

Cells and appropriate antibodies to cell surface molecules were added to standard flow cytometry staining tubes. For intracellular staining, surface stained cells were permeabilized using BD Cytofix/Cytoperm and stained with monoclonal antibodies. Cells were fixed in 2% paraformaldehyde and the samples acquired in LSR-II flow cytometer.

4 CHAPTER VI: RESULTS AND DISCUSSION

4.1 PAPER I: IMPAIRED PHENOTYPE AND FUNCTION OF PTFH CELLS IN HIV-1 INFECTED CHILDREN RECEIVING ART

Tfh cells are critical for GC formation and differentiation of GC B cells to long-lived plasma cells and memory B cells. Peripheral Tfh (pTfh) cells are the counterparts of tissue Tfh cells [294, 295]. We characterized pTfh based on the expression of CXCR5 among memory CD4+ T cells [278]. We also used the expression of CCR6 and CXCR3 chemokine receptors to identify Th1, Th2 and Th17-like Tfh cells [358]. Besides, the expressions of ICOS and PD1 were assessed on pTfh cells [359]. The aim of this study was to elucidate the distribution of pTfh cells and B cell subsets in HIV-1 infected children receiving ART.

Our finding disclosed a significantly reduced frequency of pTfh cells in HIV-1 infected compared to healthy control children (Figure 6a) in spite of the fact that the frequency of CD4+CD45RO+ memory T cells was significantly higher in HIV-1 infected children. There was no difference in pTfh cells' frequency between aviremic and viremic HIV-1 infected children (Figure 6a). Co-stimulatory molecules are critical for the interaction between Tfh and GC B cells and, accordingly, we measured the expression of ICOS and PD1 on pTfh cells. A lower frequency of pTfh cells expressing PD1, ICOS and PD1+ICOS was identified in samples from HIV-1 infected children (Figure 6b-d).

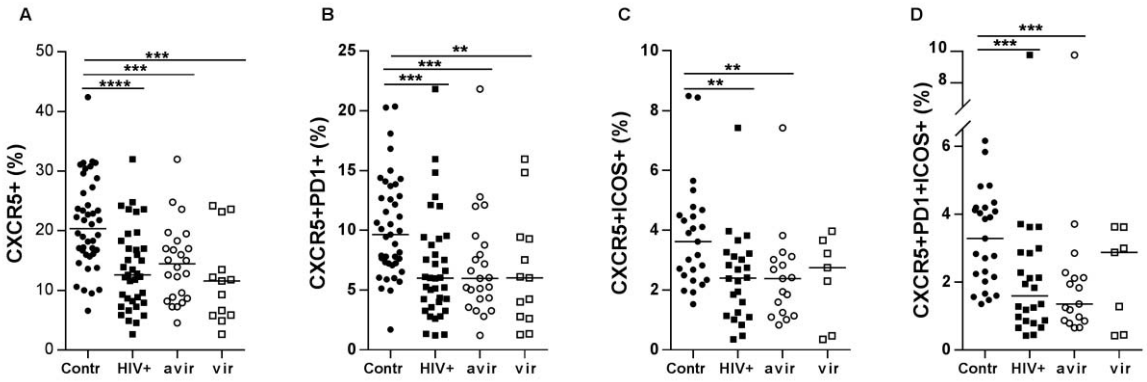

Figure 6: *Frequencies of pTfh cells (a) and PD1+, ICOS+ and PD1+ ICOS+ pTfh cells (b-d) in HIV-1 infected and healthy controls (gated on CD4+CD45RO+ cells). (Contr: controls; HIV+: ART treated HIV-1 infected; avir: aviremic; vir: viremic).*

Subsequently, we measured the expression of cytokines IFN-γ, IL-2, IL-4 and IL-21 in pTfh cells upon *in vitro* polyclonal stimulation with PMA and ionomycin in the presence of

Golgistop. A similar frequency of pTfh cells expressing IFN-γ, IL-2 and IL-21 were measured in HIV-1 infected children and healthy controls (Figure 7a, b and d); however, a reduced frequency of IL-4+ pTfh cells was observed in HIV-1 infected children (Figure 7c). IL-4 is critical for B cells survival and differentiation to antibody producing cells in response to antigens or vaccines; our finding thus suggests that, in view of the low frequency of IL-4+ pTfh cells, vaccine specific responses may be affected in HIV-1 infected children.

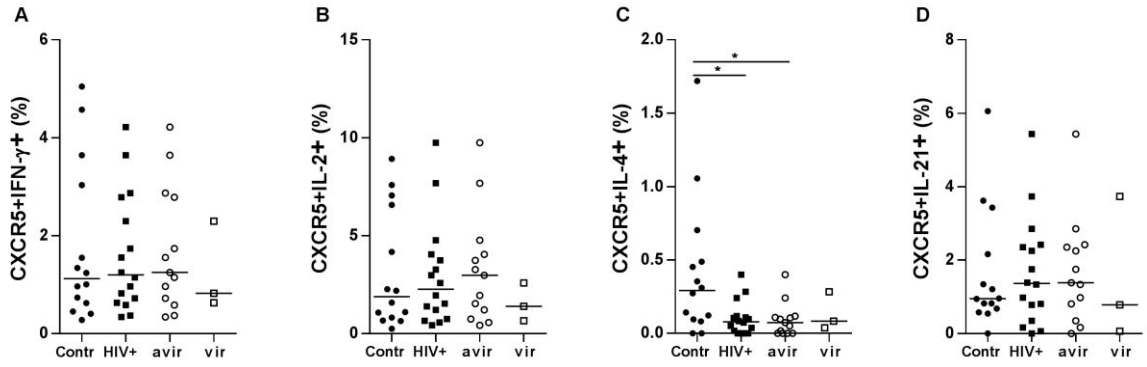

Figure 7*: Expression of cytokines (IFN-γ, IL-2, IL-4 and IL-21) in pTfh cells in heathy controls and ART treated HIV-1 infected children; pTfh cells were gated from CD4+CD45RO+ T cells. (Contr: controls; HIV+: ART treated HIV-1 infected; avir: aviremic; vir: viremic).*

In addition, B cell subsets were characterized in HIV-1 infected and controls children (Figure 8); the frequency of CD19+ B cells was reduced in HIV-1 infected children with a lower frequency observed in viremic as compared to aviremic children. The frequency of naïve B cells (CD19+CD10–CD21+CD27–) was similar between HIV-1 infected and controls; however, viremic children displayed a lower frequency of naïve B cells compared to aviremic and control children. Resting memory B cells (CD19+CD10–CD21+CD27+), critical for serological memory to vaccines and infections, were significantly reduced in HIV-1 infected children compared to healthy controls, regardless of their viral load status. A similar frequency of activated memory B cells (CD19+CD10–CD21–CD27+) was measured between HIV-1 infected and control children. Tissue-like memory B cells (CD19+CD10–CD21–CD27–) were significantly higher in HIV-1 infected compared to control children. Both populations of exhausted B cells (activated memory and tissue-like memory B cells) were significantly higher in viremic compared to aviremic and controls children (Figure 8).

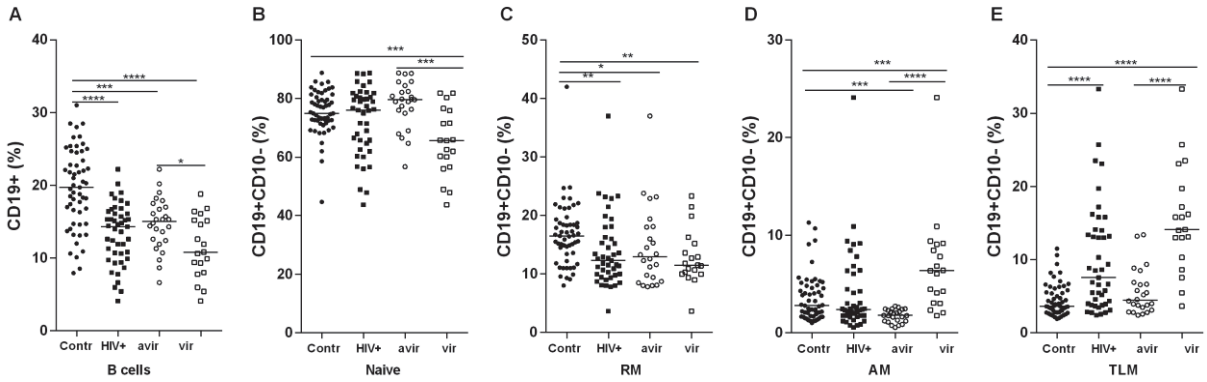

Figure 8*. B cell subsets in 45 (26 aviremic and 19 viremic) HIV-1 infected children and 55 healthy controls. (Contr: controls; HIV+: ART treated HIV-1 infected; avir: aviremic; vir: viremic; RM: resting memory; AM: activated memory; TLM: tissue-liked memory B cells; * P<0.05; ** P<0.01; ***P<0.001; ****P<0.0001).*

In **paper I,** we found profound defects in Tfh cells and B cell subsets of HIV-1 infected children who were on ART. The main reason for this could be that ART was initiated when a profound immunological damage had already taken place in this children starting from birth. ART was initiated when the CD4+ T cell counts were below 350 cells/µl (for 3-5 years old) or below 200 cells/µl (older than 5 years) and/or when the children reached the WHO clinical stages III and IV, according to the Ethiopian pediatric ART guidelines [360]. The death rate among HIV-1 infected, ART naïve children can reach up to 52% before celebrating the second year of age [361] with respiratory diseases as the major cause of death [362]. Tissue Tfh cells were expanded in chronically HIV-1 infected ART naïve adults and significantly affected B cells maturation and differentiation in GC [343]. B cell subsets and immunoglobulin production is impaired, directly or indirectly, by HIV-1 infection. Looking at the clinical records of the HIV-1 infected children enrolled in the study we found that only 26.1% started ART within 12 months from birth. Early initiation of ART reduces the destruction of CD4+ T cells and increases the longevity of memory B cells [315]. Thus, also from an immunological perspective, ART initiation should be recommended for all HIV-1 infected children from birth as found in the latest WHO guidelines [119]. We were also concerned about the ART adherence behaviors of HIV-1 infected children since they had very high viral load titers in blood at the time of sample collection. For obvious reasons, the treatment adherence of HIV-1 infected children is usually affected by their caregivers or parents [127-131]. Therefore, the finding of my study suggests that there should be a close

clinical follow-up to reduce treatment failure in children was still present with high viral load after 6 months of ART.

4.2 PAPER II: PTFH CELLS AND ANTIBODY RESPONSES TO HBV VACCINE IN HIV-1 INFECTED CHILDREN RECEIVING ART

HBV infection is a serious health problem in developing countries which can cause acute or chronic infection, with the latter leading to cirrhosis or HCC. HBV vaccine can prevent the infection and related cancer. It was previously reported that the frequency of pTfh cells correlated with the levels of antibody responses to influenza vaccination [298, 363] and to an Ebola vaccine candidate [299]. Havenar-Daughon and colleagues reported that plasma CXCL13 might be a useful marker for monitoring GCs activity during vaccination [300]. My study was carried out to characterize the contribution and response of pTfh cells during HBV vaccination of HIV-1 infected and age matched healthy controls; in addition, we asked the question on whether measurement of CXCL13 in serum would be a useful marker to monitor vaccination responses.

In the context of **paper II**, we recruited ART treated HIV-1 infected children and healthy control children who did not previously receive HBV vaccination. After screening for previous HBV exposure and current HBV infection, three doses of HBV vaccine (10 mcg/dose) were administered to all children following an accelerated vaccination schedule. HBV vaccine responses in children were measured at 1 and 6 months from the last vaccination. We measured antibody responses to HBV vaccine and CXCL13 levels in plasma. We characterized the frequency of pTfh cells prior and post vaccination and measured in vitro cytokines response of pTfh cells upon stimulation of PBMCs with HBsAg protein.

Except for four HIV-1 infected children, all individuals responded to the HBV vaccine and mounted variable antibody levels. Ten HIV-1 infected and two healthy controls had low antibody levels (1-2 log IU/L) after 1 month from the last vaccination. HIV-1 infected children had significantly lower anti-HBs levels at 1 month and 6 months from the last vaccination compared to controls (Figure 9a and b). Our result showed that accelerated HBV vaccination elicited high levels of anti-HBs in healthy controls and HIV-1 infected children at 1 month from vaccination but the levels of HBV antibody dramatically declined at 6 months from the last vaccination (Figure 9c). In fact, we found that 12.15% healthy controls and 22.13% HIV-1 infected children displayed a reduction of median anti-HBs antibody at 6 months compared

to 1 month from the last vaccination (Figure 9c). Based on the findings obtained at 6 months from vaccination, consideration should be given to implement the schedule of accelerated HBV vaccination with a fourth vaccine dose to slow down the rapid decline in the antibody levels and to increase the longevity of HBV vaccine response in resource-limited countries. It has been recommended by authorities to introduce a fourth HBV vaccine dose at 12 months of age in the accelerated vaccination schedule [194]. Yet, most African countries have not followed the recommendation due to logistic and vaccine compliance issues.

Studies have previously shown that HIV-1 RNA copies transiently increased following influenza vaccination [364, 365] whereas other publications have not reported any change in this context [366, 367]. In our study, there was no significant change in the number of HIV-1 RNA copies after HBV vaccination (data not shown).

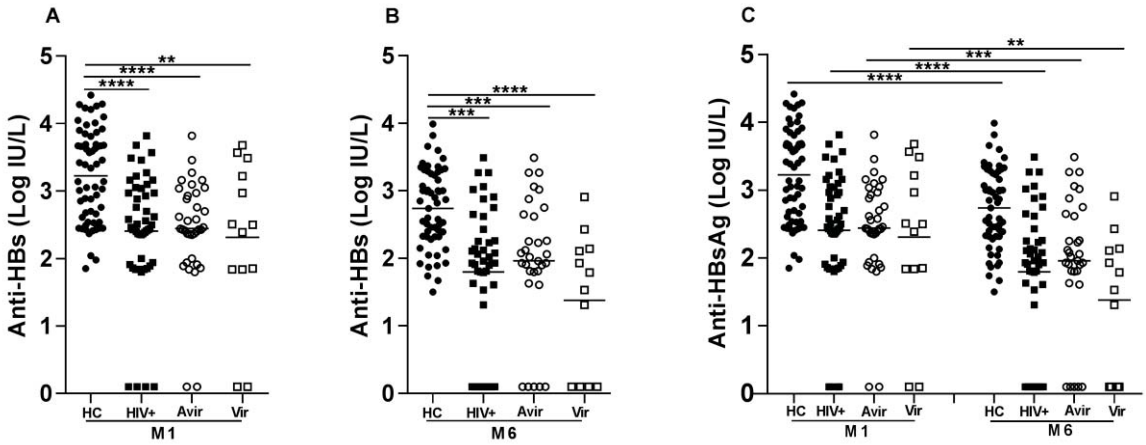

Figure 9*. Plasma levels of anti-HBs after 1 and 6 months from the last vaccination in HIV-1 infected children and healthy controls (HC: healthy controls; HIV+: ART treated HIV-1 infected; avir: aviremic; vir: viremic; BL: baseline; M1: 1 month after the last vaccination; M6: 6 months after the last vaccination).*

The frequency of pTfh cells was similar before and after vaccination in HIV-1 infected subjects and healthy controls (Figure 10a). The expression of CXCR3 and CCR6 in pTfh cells gave us the opportunity of characterizing Th1, Th2 and Th17-like pTfh cells [358]. In our study, an elevated frequency of Th17-like pTfh cells (CXCR3-CCR6+) was observed in HIV-1 infected children compared to healthy controls prior to vaccination while no difference was observed after 1 month from vaccination. Interestingly, healthy controls exhibited a significant change in the frequency of Th17-like pTfh cells at 1 month post-vaccination compared to baseline (Figure

10b). As Th17-like pTfh cells engage in immunity against infectious diseases, including HIV-1 infection, and autoimmune diseases [368, 369], these cells might possibly also be important for vaccine induced memory [299]. We have also found an inverse correlation between the CD4+ T cell count and the frequency of Th17-like pTfh cells in HIV-1 infected children prior to vaccination (Figure 10c) suggesting a possible immunopathological link between disease progression and frequency of Th17-like pTfh cells. On the contrary to what we had shown in **paper I** where the frequency of pTfh cells was significantly lower in HIV-1 infected subjects compared to controls, in **paper II**, there was no difference in the frequency pTfh cells between HIV-1 infected and healthy controls. Based on WHO recommendations, in 2014 ART treatment was provided to all children younger than 15 years of age, regardless of their CD4+ T cells count and clinical stage [370]. The cohort of HIV-1 infected children in **paper II** was enrolled in 2015 and most of the children were on ART before their CD4+ T cells severely declined, thus avoiding the development of HIV-1 related complications and AIDS-defining illnesses in this group. It is therefore possible that ART normalized the frequency of pTfh cells in HIV-1 infected children who were enrolled in **paper II**.

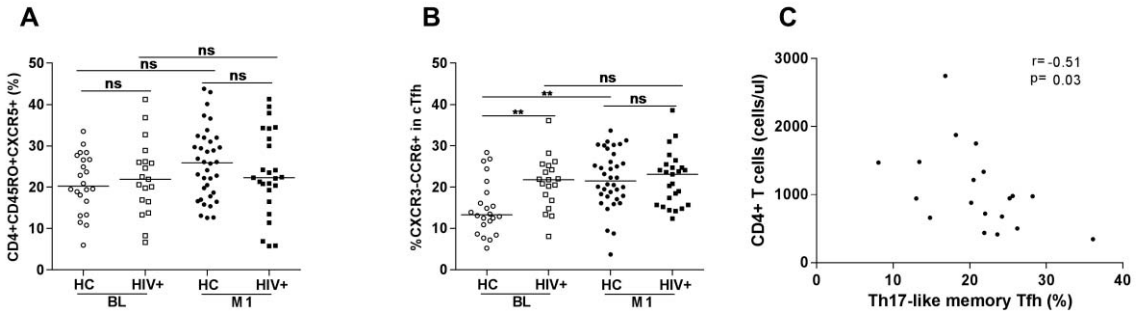

Figure 10*. Frequency of pTfh cells (a), expression of CXCR3 and CCR6 in pTfh cells (b) and correlation of Th17-like pTfh cells with CD4+ T cells. (HC: healthy controls; HIV+: ART treated HIV-1 infected; BL: baseline; M1: 1 month after the last vaccination).*

We then measured the frequencies of pTfh cells expressing the cytokines IFN-γ, IL-2, IL-4 and IL-21 after stimulation of PBMCs for 5 days in presence of the CD28/CD49d costimulatory complex alone or together with the HBsAg protein. Similar expression levels of cytokines were observed between HIV-1 infected and healthy controls at baseline and at 1 month post-vaccination. The expression of cytokines was significantly increased in both groups at 1 month post-vaccination compared to baseline in PBMCs stimulated with HBsAg protein together with co-stimulatory molecules but not cultures treated with the co-costimulatory complex alone

(Figure 11). In **paper II**, we are able to set-up a new protocol based on our previous polyclonal stimulation experiments **(paper I)** and a study conducted on CD4+ T cell response upon HBsAg protein stimulation [371]. In our experiments, it was striking that pTfh cells from HIV-1 infected and healthy controls expressed similar levels of cytokines pivotal for Tfh cell biology.

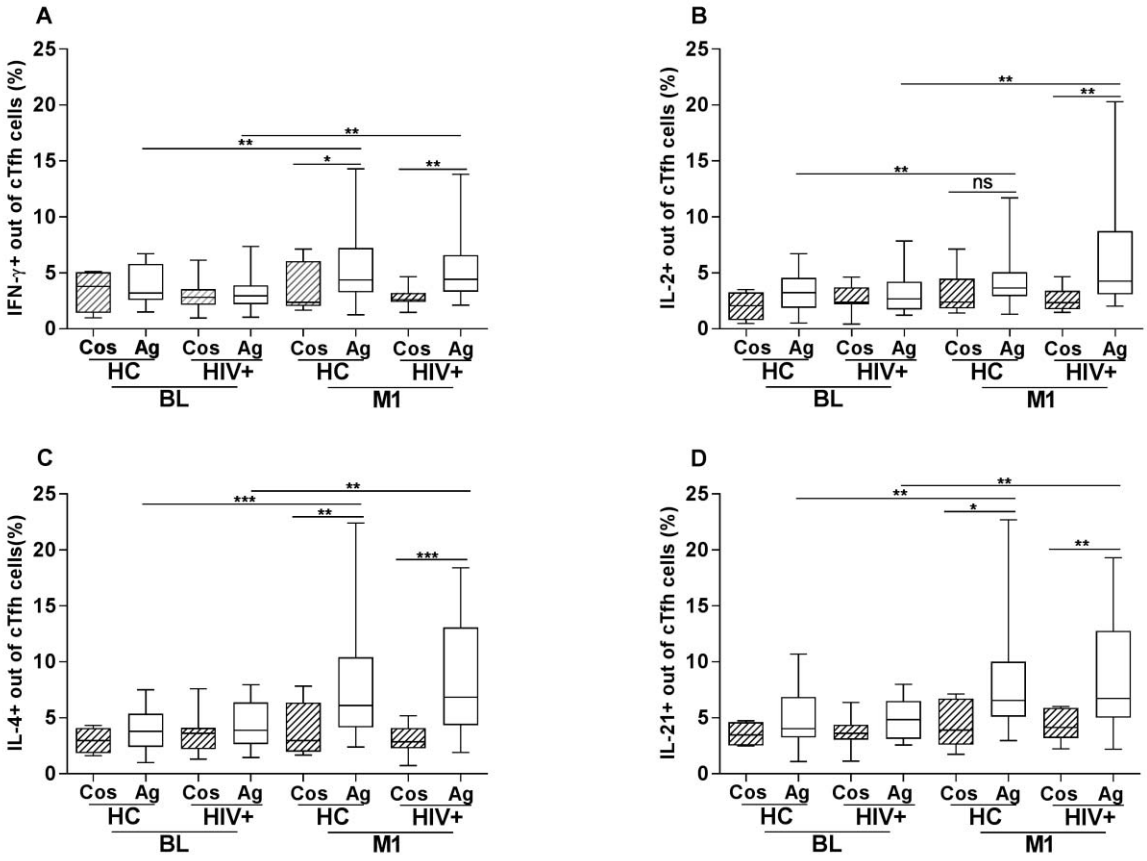

Figure 11. *Expression of cytokines (IFN-γ, IL-2, IL-4 and IL-21) by pTfh cells after PBMCs stimulation with HBsAg protein together with co-stimulatory molecules (Ag) or co-costimulatory molecules alone (Cos) for 5 days. (HC: healthy controls; HIV+: ART treated HIV-1 infected; avir: aviremic; vir: viremic; BL: baseline; M1: 1 month after the last vaccination)*

The levels of CXCL13 were significantly higher in HIV-1 infected children at all studied time points compared to healthy controls. On this aspect, other related studies have shown similar result in HIV-1 infected individuals [305, 306, 372, 373]. There was a similar plasma CXCL13 concentration at different time points within the groups of controls and HIV-1 infected children; however, viremic children had higher levels of CXCL13 compared to aviremic at 6 months from vaccination (Figure 12). CXCL13 levels were unchanged after vaccination in both HIV-1

infected children and healthy controls. Hence, our study does not support the possibility of using CXCL13 plasma levels as biomarkers for GC activities [300]; it is however important to assess the role of CXCL13 as biomarker of successful vaccination in larger cohorts.

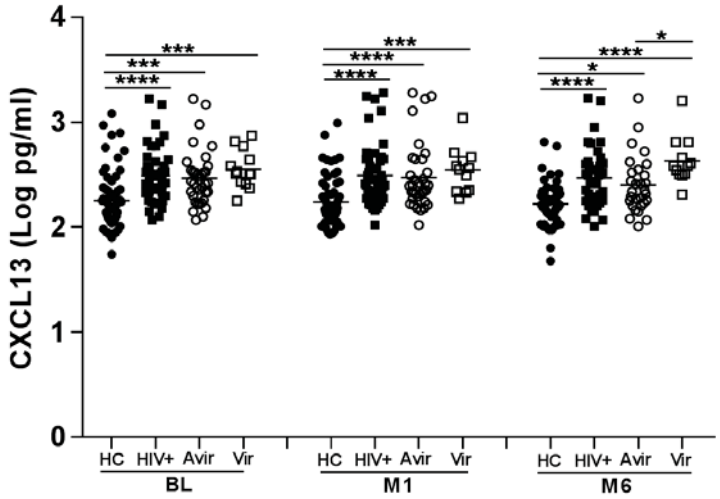

Figure 12*. Plasma CXCL13 levels in healthy controls and HIV-1 infected children (viremic and aviremic) prior to vaccination and at 1 month and 6 months from last vaccination dose (HC: healthy controls; HIV+: ART treated HIV-1 infected children; avir: aviremic; vir: viremic; BL: baseline; M1: 1 month after the last vaccination; M6: 6 months after the last vaccination).*

In **paper II**, the phenotype and function of pTfh cells were similar between HIV-1 infected children and age-matched healthy controls. High plasma levels of anti-HBs were elicited with accelerated HBV vaccination schedule. We also demonstrated a rapid decline in the levels of anti-HBs over time in HIV-1 infected and healthy controls. However, we were unable to provide a possible mechanism for the reduced levels of plasma anti-HBs in HIV-1 infected children which would be based on frequency and function of pTfh cells. In **paper II** the frequency and properties of B cell subpopulations were not examined; in the following chapters summarizing unpublished results I present results correlating HBV antibody response with B cell subpopulations.

4.3 UNPUBLISHED RESULTS: WHY IS THERE A DISCREPANCY BETWEEN THE FREQUENCIES OF PTFH CELLS MEASURED IN PAPER I AND PAPER II?

Further analyses were conducted to understand the differences in pTfh cell frequencies in **papers I** and **II**. In **paper II,** the frequency of pTfh cells was similar between HIV-1 infected

patients and healthy controls; this observation was different from **paper I** where a reduced frequency of pTfh cells was identified in HIV-1 infected children compared to the controls. Other studies also showed a similar frequency of pTfh cells in HIV-1 infected individuals and controls [340, 374]. One possible explanation for the differences noticed between **papers I** and **II** could be due to the degree of HIV-1 replication; in fact in **paper I** the numbers of HIV-1 RNA copies in plasma was significantly higher than **paper II** (Figure 13a). Moreover, there was a significant negative association between pTfh cells and HIV-1 RNA copies (Figure 13e). Boswell and colleagues showed that the frequency of pTfh cells was greatly affected by high HIV-1 RNA copies [336].

Guidelines for the timing of ART initiation in HIV-1 infected children were changed during the study period as discussed in the result section of **papers I** and **paper II.** The median length of ART treatment in the cohort of HIV-1 infected children included in **paper II** was 41.8 months. In **paper I**, the median length of ART treatment was 37 months. According to these clinical observations showing that the length of ART treatment did not differ between the two groups of HIV-1 infected children, it is difficult to envisage that the different frequencies of pTfh cells in the two studies should be related to parameters of ART administration.

In HIV-1 infected children, the frequency of pTfh cells was higher in **paper II** compared to **paper I** (Figure 13b). The median age of the children in **paper II** was significantly higher than in **paper I** both for HIV-1 infected children (Figure 13c) and healthy controls (Figure 13f). In addition, there was a direct correlation between the frequency of pTfh cells and age in HIV-1 infected children (Figure 13d). Thus, age could be another factor for the differences noticed in the frequencies of pTfh cells of HIV-1 infected children in the two studies. However, similar frequencies of pTfh cells (Figure 13f) were observed in the control groups despite an increase in age (Figure 13g) in **paper II** (S2); there was no correlation between age and pTfh cells for controls (Figure 13h).

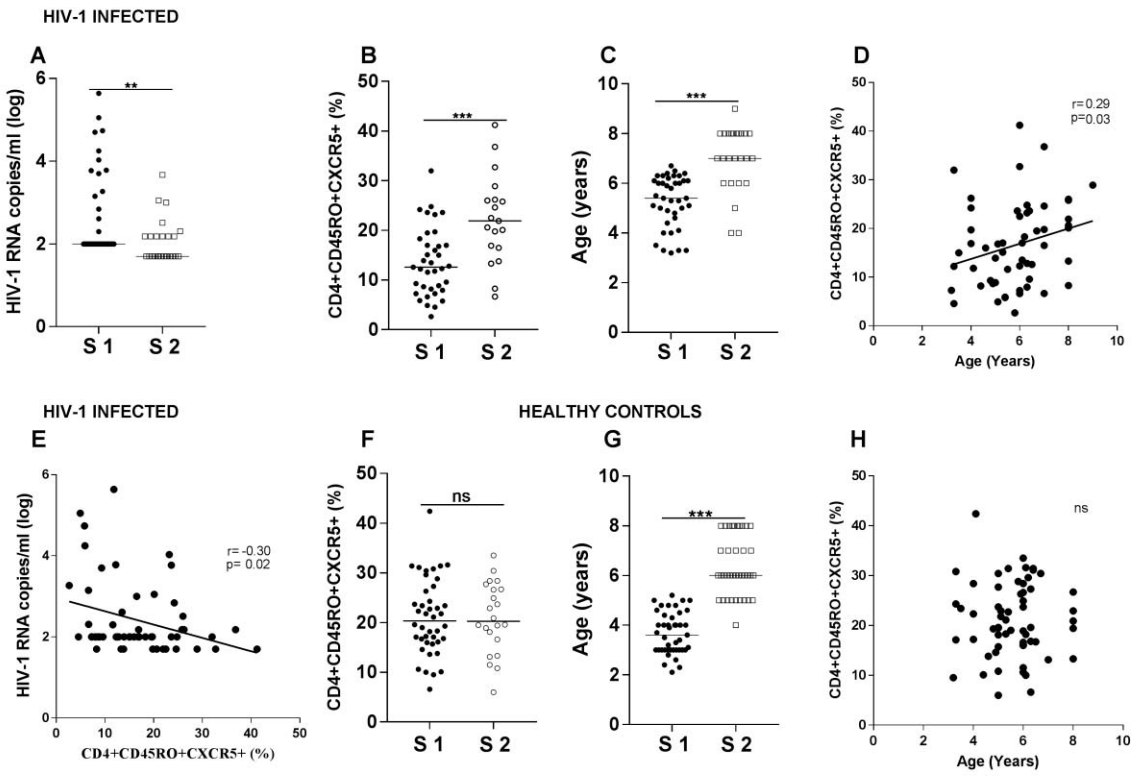

Figure 13*. Viral load, frequency of pTfh cells and age in HIV-1 infected children (panels a-e) and controls (panels f-h) enrolled in the studies of papers I and II. Study 1 (S1) and study 2 (S2).*

4.4 PAPER III: HBV VACCINATION IN HIV-1 INFECTED YOUNG ADULTS: A TOOL TO REDUCE THE SIZE OF HIV-1 RESERVOIRS?

The persistence of HIV-1 in resting memory T cells is one of the major obstacles for HIV-1 eradication. HIV-1 latency is established during the primary phase of the infection and the virus persists in long-lived memory T cells. Early initiation of ART reduces the size of the virus reservoirs but cannot eliminate the provirus DNA from the host [375]. Several clinical trials have been conducted to induce the provirus from the latently infected cells, allowing ART to eliminate the induced viruses; this approach is known as "shock and kill" [376].

We extensively studied pTfh cells in **papers I and II,** and serological memory to HBV vaccination in **paper II**. We were keen on studying other subsets of CD4+ T cells including CD8+ T cells, and the expressions of activation and proliferation markers on these cells in relation to hepatitis vaccination and the size of HIV-1 reservoirs measured as total HIV-1 DNA copies in PBMCs **(paper III)**. A couple of studies showed that CD8+ T cells play a critical role in the clearance of HBV during the acute phase of the infection [167, 168]. A recent

report showed that HIV-1 specific cytotoxic T lymphocytes were decisive in clearing the virus reservoirs in pediatric HIV-1 infection [377]. Thus, we investigated the relation between CD8+ T cells and HIV-1 DNA reservoirs in HIV-1 infected children vaccinated for HBV and HAV.

Twenty-two HIV-1 infected children were recruited to this study **(paper III)**. Protective anti-HBs (>1 IU/L) were elicited after three doses of accelerated hepatitis vaccination (Figure 14a) in all but two children. The size of the virus reservoirs, measured by HIV-1 DNA copies/10^6 PBMCs (Figure 14b), was similar prior and post vaccination. However, 54% of the patients (Figure 14c) had reduced HIV-1 DNA copies in PBMCs at 1 month from the last vaccination as compared to the baseline. We could not confirm that this result was entirely due to the effect of hepatitis A and B vaccination as a control group of aged matched HIV-1 infected children not receiving vaccination was missing in the study. Previous studies showed that the change in HIV-1 DNA copies was 1 log in 1 year and 0.2 log between 1-4 years of ART in HIV-1 infected children [378]. In a different study, adults showed a fast decay (86%) of HIV-1 DNA following 1 year of ART and 23% decay between 1-4 years; the HIV-1 DNA decay reached a plateau after 4 years of ART [379]. As the HIV-1 infected children included in **paper III** had been on ART for a median period of 7.2 years, it is likely that a plateau already reached for the decline of HIV-1 DNA copies due to ART prior to vaccination conducted in this study. Thus, immunological events related to hepatitis vaccination, were likely leading to a change in the number of HIV-1 DNA copies.

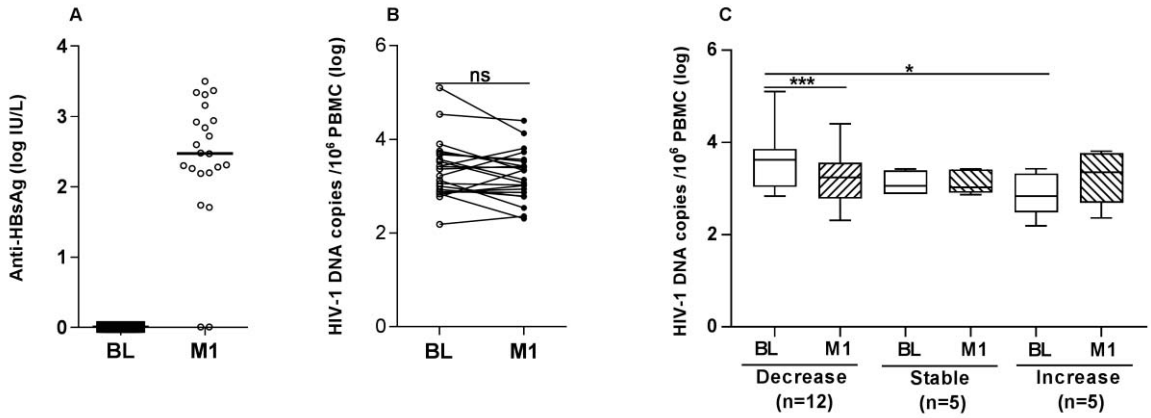

Figure 14*. Plasma levels of anti-HBs (a), HIV-1 DNA copies (b) and difference in HIV-1 DNA copies in three groups (c) prior and post vaccination (BL: baseline; M1: 1 month after the last vaccination; Decrease: > 10% reduction in HIV-1 DNA copies/10^6 PBMCs; Stable: < 10% increase or decrease in HIV-1 DNA copies; Increase: > 10% increase in HIV-1 DNA copies).*

Table 2 summarizes the differences in frequency of CD4+ and CD8+ T cell subpopulations and expression of activation markers prior and post-vaccination. The frequency of naïve CD4+ T cells was reduced following vaccination whereas the frequency of EM CD4+ T cells increased at 1 month from vaccination compared to baseline. The frequency of CM CD4+ T cells expressing CD38 was significantly reduced at 1 month. CM CD8+T cells increased significantly; the frequencies of cells expressing HLA-DR declined among total, CM and EM CD8+ T cells. The expression of Ki67 was significantly reduced among CM and EM CD4+ T cells and in the total, EM and TEMRA CD8+ T cells at 1 month post-vaccination. Immune activation and proliferation is the driving force for disease progression in HIV-1 pathogenesis [380]. Our data showed reduced immune activation and proliferation of CD4+ and CD8+ T cells post vaccination with Twinrix.

Table 2. CD4+ and CD8+ T cell subsets and their surface expression of activation and proliferation markers in ART treated young children prior and post vaccination.

T cells	Difference in CD4+ T cell subsets between BL and M1				Difference in CD8+ T cell subsets between BL and M1			
	CD4+	CD38	HLA-DR	Ki67	CD8+	CD38	HLA-DR	Ki67
Total							↓*	↓*
Naïve	↓**							
CM		↓**		↓**	↑**		↓*	
EM	↑*			↓**			↓**	↓**
TEMRA								↓*

(BL: baseline; M1: 1 month after the last vaccination; CM: central memory; EM: effector memory; TEMRA: Terminally differentiated effector memory).

We further analyzed T cells in patients who had lower HIV-1 DNA copies following vaccination and also the two other groups in which HIV-1 DNA was stable or increased; the frequency of Ki67+ expressing cells among CM and EM CD4+ T cells (Figure 15a and b) was reduced whereas the frequency of CM CD8+ T cells (Figure 15c) was increased at 1 month post-vaccination. There was no change in the frequency of CD4+ and CD8+ T cell subsets in the stable and increase groups of patients.

A previous publication reported that HIV-1 infected individuals with low viral load showed low levels of immune activation and proliferation of CD4+ T cells [381]. A study conducted to predict the half-life of T cells in HIV-1 infected patients showed that CM CD8+ T cells had a

shorter half-life (50 days) compared to healthy individuals (100 days). Loss of CM CD8+ T cells during HIV-1 infection could be due to a declined expression of IL-7Rα, a pathogenic phenomenon of both CD4 and CD8 T cell which was previously linked to disease progression [382, 383]. Thus, reduced proliferation levels of CD4+ T cells and increased CM CD8+ T cells in the "decreased" group of HIV-1 infected young adults post-vaccination may be part of the mechanism leading to a reduction of HIV-1 DNA copies observed in some children upon vaccination with Twinrix.

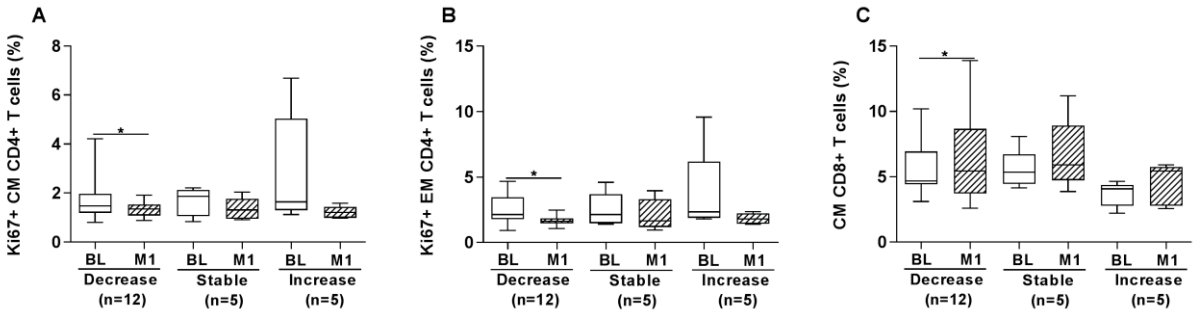

Figure 15. *Frequency of CD4+ and CD8+ T cells and Ki67 expression on subpopulations of CD4+ T cells in 'decrease', 'stable' and 'increase' groups of HIV-1 infected young adults prior and post HBV vaccination (BL: baseline; M1: 1 month after the last vaccination; Decrease: > 10% reduction in HIV-1 DNA copies/10^6 PBMCs; Stable: < 10% increase or decrease in HIV-1 DNA copies; Increase: > 10% increase in HIV-1 DNA copies).*

Linear regression analysis was performed and a model established to predict the contribution of clinical and immunological parameters to the changes of HIV-1 DNA copies between the two time points. We first selected parameters which could predict HIV-1 DNA changes by using univariate analysis. Next, stepwise multivariate linear regression analysis was used: through these calculations we found that 75% of the variance for the decline of HIV-1 DNA after vaccination was explained by the frequency of EM CD8+ T cells.

A role for functional CD8+ T cell responses has been shown for both HBV and HIV-1 infections. In mice, HBV vaccine elicited a strong and broad CD8+ T cell response which can be of use for treatment of chronic HBV infection [384-386]. Moreover, HBV-specific EM CD8+ T cells were high in an individual who cleared HBV acute infection [170]. A study conducted to assess immune responses upon co-delivery of SIV gag DNA vaccine with IL-12 showed that EM CD8+ T cells increased significantly in the vaccinated macaques [387]. Viral load increased upon depletion of CD8+ T cells with an anti-CD8+ monoclonal antibody

suggesting the role of CD8+ T cells in suppressing the virus from circulation [387]. HIV-1 specific CD8+ T cells reduced the seeding of virus reservoirs in the acute phase of HIV-1 infection and there was an inverse correlation between HIV-1 DNA copies in PBMCs and frequency of effector CD8+ T cells [388]. EM CD8+ T cells were strongly inhibiting SIV replication after mucosal challenge of vaccinated macaques [389, 390]. *In vitro* experiment of sorted EM CD8+ T cells co-cultured with HIV-1 infected CD4+ T cells showed a strong and consistent inhibition of viral replication from cells obtained from elite controllers [391].

In summary, HBV vaccination could be a potential candidate strategy to reduce the size of virus reservoirs in successfully ART treated patients. This study has the following limitations: the number of HIV-1 infected children and young adults in Sweden is very low and finding non-HBV vaccinated children was not possible; accordingly we cannot firmly claim that the change in HIV-1 DNA copies detected post vaccination was due to vaccination.

4.5 PRELIMINARY RESULTS: B CELL SUBSETS OF HIV-1 INFECTED CHILDREN.

There was an open question in **paper II** that should be addressed to explain the differences in the anti-HBs levels in healthy controls and HIV-1 infected children. B cells are important components in the successful response to vaccines. In this section of **preliminary results**, I explored the possibility that impairments in frequencies of B cell subsets could account for the poor serological response to HBV vaccine in HIV-1 infected children reported in **paper II**.

The results from these analyses presented in Figure 16 are in line with several previous studies which reported a dramatic alteration in B cell subsets of HIV-1 infected children [315, 330, 334, 392, 393]. A significantly reduced frequency of Naïve, RM and switched B cells (Figure 16C, 16D and 16G) was found in HIV-1 infected children compared to healthy controls; on the contrary, the frequencies of AM and TLM B cells, exhausted B cell subsets, were significantly increased in HIV-1 infected children (Figure 16E and F). ART administration contributed to restore the frequencies of RM B cells (Figure 16D) and switched B cells (Figure 16G) whereas treatment had no impact on the elevated frequencies of AM (Figure 16E) and TLM (Figure 16F) B cells.

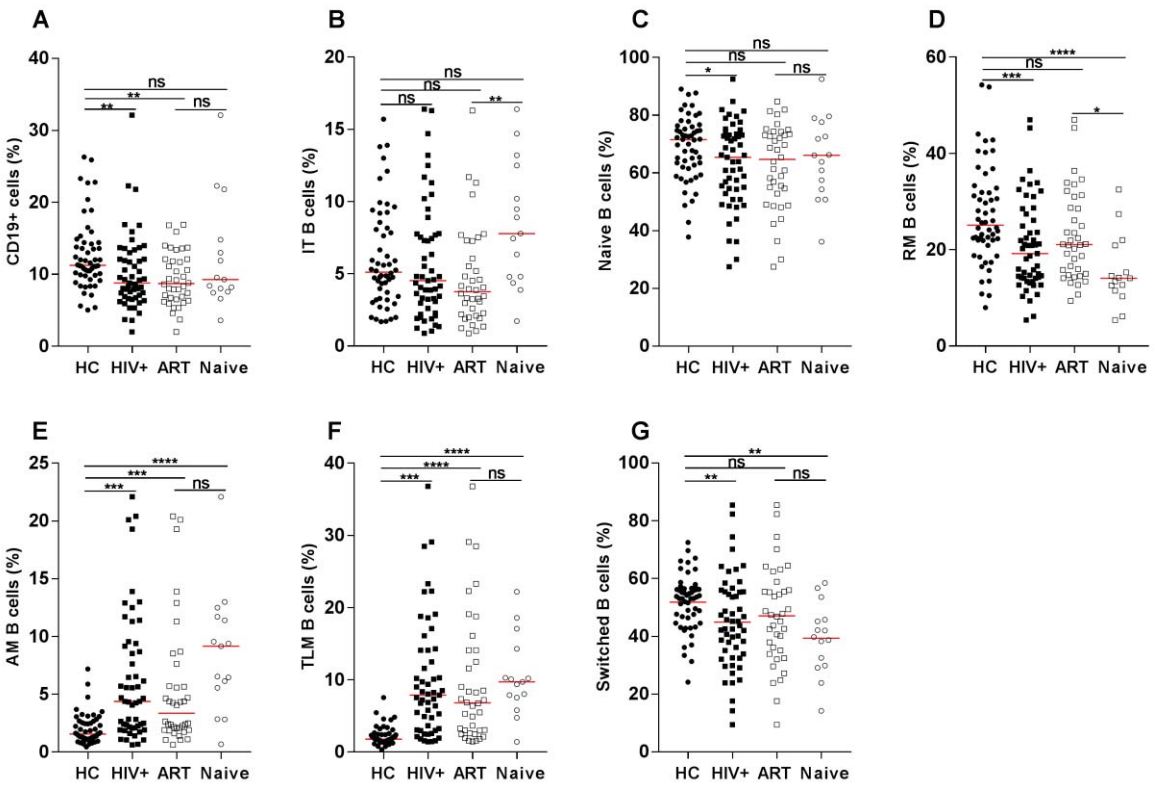

Figure 16. *Frequency B cell subsets in healthy controls and HIV-1 infected (ART treated and naive) children (HC: healthy controls; HIV+: HIV-1 infected; ART: ART treated HIV-1 infected; Naïve: ART naïve HIV-1 infected; IT: immature transitional B cells; RM: resting memory; AM: activated memory; TLM: tissue-liked memory; ns: not significant; * P<0.05; ** P<0.01; ***P<0.001; ****P<0.0001).*

We calculated the correlation between the frequencies of B cell subsets and the level of anti-HBs antibodies and found a significant correlation of anti-HBs antibodies with RM B cells (r=0.56; p<0.0001) and switched memory B cells (r=0.35; p=0.02) in HIV-1 infected children (Figure 17d and g). There was no correlation between anti-HBs antibody and B cell subsets in healthy controls.

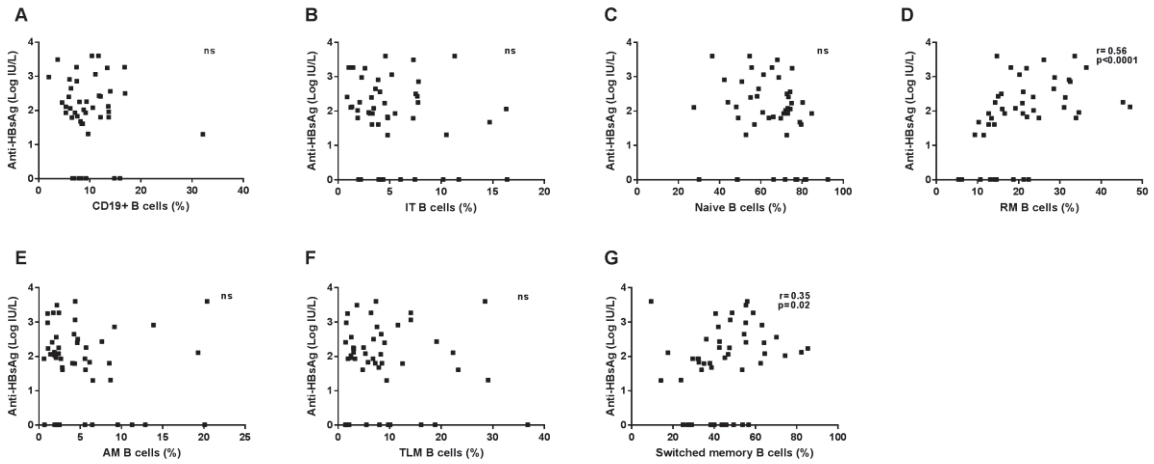

Figure 17. Correlation between plasma titers of anti-HBs antibody and B cell subsets in HIV-1 infected children. (*IT: immature transitional B cells*; *RM: resting memory; AM: activated memory; TLM: tissue-liked memory; ns: not significant).*

It is evident from these findings that the poor HBV vaccine response observed in **paper II** was mostly likely due to defects existing in the B cell compartment. ART provided to HIV-1 infected children is critical to improve the distribution and function of B cells. Perturbation of B cell subsets, however, can still be found in ART treated children, which may affect vaccine specific memory B cells and long-lived plasma cells.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

Vaccines are the most effective interventions to control infectious diseases and save millions of lives globally. More than 250 thousand people are chronic HBV carriers and thousands of lives are lost each year globally due to HBV related complications. HBV infection is preventable and a vaccine with 95% efficiency is available [140]. A poor response to HBV vaccine was reported by several studies in HIV-1 infected individuals and protective HBV antibodies induced by vaccination wane faster in HIV-1 infected individuals than in healthy controls.

Tfh cells and RM B cells are critical components of effective vaccine responses. In **paper I**, we showed that HIV-1 infected children had lower frequencies of pTfh and RM B cells compared to healthy controls. As the majority of children included in this study were administered ART after one year of age, it is likely that the disruption, or lack of development of immune components relevant for response to vaccines occurred in the children before the initiation of ART. Thus, early initiation of ART is mandatory to preserve humoral and cellular immune responses in children. In **paper II**, higher plasma levels of anti-HBs were measured in healthy controls compared to infected children; antibody responses to the HBV vaccine declined in both groups after 6 months. This finding poses a question regarding the timing of accelerated vaccination schedule and the importance of adjusting the schedules for HIV-1 infected individuals. In addition, the introduction of a fourth dose of HBV vaccine should be assessed in the context of resource-limited settings [194]. It would be interesting to further follow the HBV vaccine responses in the children enrolled in **study II** to measure the decay in plasma anti-HBs levels after 5 years; these data, together with the ones which we have already collected, could be used to calculate the decay of humoral immune responses to HBV vaccine over time using mathematical modeling.

In **paper II,** we set-up a protocol to measure cytokine expression in pTfh cells upon stimulation of PBMCs with the HBsAg protein. In the future, I also would like to perform experiments to evaluate the phenotypic and functional characteristics of HBsAg specific CD8+ T cells, a parameter that was not evaluated in HIV-1 infected children in our previous studies. To assess cytokine expression of HBsAg specific CD8+ T cells may give the opportunity of better understanding the role of CD8+ T cells in HBV vaccination; CD8+ T cells are crucial for clearance of the virus during acute infection and they are involved in different stages of chronic HBV infection [168, 169]. The characteristics of HBsAg specific memory B cells should also be studied in this context in healthy and HIV-1 infected children as they play an important role in serological memory to HBV vaccine. Based on a recent publication from another group

[300], the plasma concentration of CXCL13 was proposed to represent a marker of ongoing GC activities. In our study, the plasma concentration of CXCL13 was elevated in HIV-1 infected children at all time points and changes in CXCL13 levels were not observed after vaccination in either group. Several studies reported that elevated plasma CXCL13 concentration can be found during HIV-1 infection, with activated monocytes heavily involved in its production [305, 306, 372, 373]. Further studies should be conducted to elucidate the use of CXCL13 as a GC activity marker and the possibility of using CXCL13 to predict the emergence of cross-neutralizing antibodies in HIV-1 infected individuals [331].

The scale-up of ART in Ethiopia was a timely intervention since early ART initiation mitigates immune activation and reduced morbidity and mortality in the patients. A continuous clinical follow-up of HIV-1 infected children should be conducted to improve the rate of adherence, to monitor treatment failure, to reduce drug related toxicity and ART drug resistance [394]. High circulating levels of HIV-1 RNA were detected in some children receiving ART who were enrolled in study I and II of this PhD thesis; accordingly, the rate of ART adherence and associated factors should be investigated in HIV-1 infected children in ART clinics of Addis Ababa and other regions of Ethiopia.

In **Paper III**, we showed a possible connection between vaccination against HBV and the number of HIV-1 DNA copies in some HIV-1 infected young adults included in a perspective vaccination study. There was a similar number of HIV-1 DNA copies pre- and post-vaccination in HIV-1 infected young adults included in the study; however, in 54% of these patients there was a significant reduction in HIV-1 DNA copies after vaccination. After examining the literature in relation to the decay of HIV-1 DNA copies in PBMCs in adults and children infected with HIV-1, we concluded that a concrete possibility exists that the change in HIV-1 DNA copies, representing a measure of HIV-1 reservoir, may be due to HBV vaccination. For a better understanding of this observation, I would like to conduct further studies in a large cohort of Ethiopian adults by including ART treated patients and healthy controls, previously vaccinated or not with HBV vaccines; these cohorts will be useful to assess the effect of ART on T cell subsets frequencies and changes in HIV-1 DNA after vaccination.

Our preliminary results showed that one of the reasons for the low levels of anti-HBs antibody in HIV-1 infected children following vaccinations might be the damage occurring to B cell subsets, including RM B cells. As previously studied for other childhood vaccines [315], I would like to evaluate, in a longitudinal study, the frequency of B cell subsets in HIV-1 infected

children treated from birth in Ethiopia and relate the B cell data to childhood vaccine responses. These studies could help understanding the dynamics of normalization of B cell subsets as well as the longevity of vaccine specific antibody responses in HIV-1 infected children. These studies may also be important to support changes in HBV vaccination schedule to improve vaccination of HIV-1 infected children.

In conclusion, we were able to elicit protective anti-HBs antibodies after three doses of HBV vaccine using an accelerated vaccine schedule. However, a fast decay of these antibodies was noticed in HIV-1 infected children, and to some extent healthy control children, which raises the important question on whether these individuals will be protected, in the long run, from hepatitis infection and related complications. HIV-1 infected children should be followed regularly and booster doses should be administered before the protective HBV antibodies wane from circulation.

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