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DYSFUNCTIONAL PHENOTYPE OF T CELLS AND THEIR CONTRIBUTION TO IMPAIRED B CELL FUNCTION DURING HIV-1 INFECTION

Rebecka Lantto Graham

Karolinska Institutet

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Dysfunctional phenotype of T cells and their contribution to impaired B cell function during HIV-1 infection

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Rebecka Lantto Graham

Principal Supervisor:
Professor **Francesca Chiodi**
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology

Co-supervisor(s):
Associate Professor **Bence Rethi**
Karolinska Institutet
Department of Medicine, CMM

Associate Professor **Anna Nilsson**
Karolinska Institutet
Department of Women's and Children's Health

Dr **Sylvie Amu**
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology

Opponent:
Professor **Ana E. Sousa**
Universidade de Lisboa
Faculty of Medicine
Institute of Molecular Medicine

Examination Board:
Associate Professor **Annika Karlsson**
Karolinska Institutet
Department of Laboratory Medicine

Professor **Sandra Kleinau**
Uppsala University
Department of Cell and Molecular Biology

Associate Professor **Charlotta Nilsson**
Karolinska Institutet
Department of Laboratory Medicine

To my family

LIST OF SCIENTIFIC PAPERS

I. Ruffin N, **Lantto R**, Pensieroso S, Sammicheli S, Hejdeman B, Réthi B, Chiodi F. *Immune activation and increased IL-21R expression are associated with the loss of memory B cells during HIV-1 infection.* J Intern Med. 2012 Nov;272(5):492-503. doi: 10.1111/j.1365-2796.2012.02550.x.

II. Sammicheli S, Ruffin N, **Lantto R**, Vivar N, Chiodi F, Réthi B. *IL-7 modulates B cells survival and activation by inducing BAFF and CD70 expression in T cells.* J Autoimmun. 2012 Jun;38(4):304-14. doi: 10.1016/j.jaut.2012.01.012.

III. **Lantto R**, Nasi A, Sammicheli S, Amu S, Fievez V, Moutschen M, Pensieroso S, Hejdeman B, Chiodi F, Réthi B. *Increased extrafollicular expression of the B-cell stimulatory molecule CD70 in HIV-1 infected individuals.* AIDS. 2015 Sep 10;29(14):1757-66. doi: 10.1097/QAD.0000000000000779.

IV. Amu S*, **Lantto Graham R***, Bekele Y, Nasi A, Bengtsson C, Réthi 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.
*shared first authorship

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Bekele Y, Amu S, Bobosha K, **Lantto R**, Nilsson A, Endele B, Gebre M, Aseffa A, Réthi 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.

Kiene M, Réthi B, Jansson M, Dillon S, Lee E, **Lantto R**, Wilson, Pöhlmann S, Chiodi F. *Toll-like receptor 3 signalling up-regulates expression of the HIV co-receptor G-protein coupled receptor 15 on human CD4+ T cells.* PLoS One. 2014 Feb 18;9(2):e88195. doi: 10.1371/journal.pone.0088195. eCollection 2014.

Sammicheli S, Dang VP, Ruffin N, Pham HT, **Lantto R**, Vivar N, Chiodi F, Réthi B. *IL-7 promotes CD95-induced apoptosis in B cells via the IFN-γ/STAT1 pathway.* PLoS One. 2011;6(12):e28629. doi: 10.1371/journal.pone.0028629.

ABSTRACT

Microbial translocation and increased immune activation have been involved in functional T cell impairments and disease progression during HIV-1 infection. The impact of microbial translocation on the phenotype of memory B cells in HIV-1 infected patients was studied in **paper I**. The expression of activation marker IL-21R was higher in HIV-1 infected patients compared with controls. An inverse correlation was observed between IL-21R expression and frequency of resting memory (RM) B cells in blood; IL-21R+ RM B cells were more sensitive to apoptosis and their frequency correlated with sCD14, a marker of microbial translocation. Furthermore, TLR triggering by microbial products resulted in IL-21R expression on memory B cells *in vitro*. These results suggest a direct link between microbial translocation and an impaired B cell phenotype.

In paper II we showed that IL-7 induced upregulation of CD70 expression on T cells. Increased CD70 expression, by triggering the CD27 receptor on B cells, can lead to alteration of the B cell phenotype and IgG production. In addition, IL-7 led to an increased production of BAFF by T cells, which enhanced B cell survival in vitro. In the context of HIV-1 infection, the mechanisms mediated by increased CD70 expression on T cells might be implicated in establishment of B cell activation, a characteristic of immune pathology in infected patients. The role of CD70 in B cell dysfunction during HIV-1 infection was further studied in **paper III**. We found an increased expression of CD70 on CD4+ T cells which correlated with CD4+ T cell depletion and viremia in HIV-1 infected patients. CD4+ CD70+ T cells expressed pro-inflammatory cytokines and, based on their chemokine profile, it was predicted that they can migrate to sites of inflammation. A potential role for CD4+ CD70+ T cells in B cell activation in HIV-1 infected individuals was suggested by the association with CD38 and CD95 expression in memory B cells, with increased B cell proliferation and plasma IgG levels. The mechanism leading to CD70 up-regulation on T cells during HIV-1 infection remains elusive.

Although treatment with ART can lead to a nearly complete suppression of HIV-1 replication, ART does not fully target the increased immune activation found in HIV-1 infected patients. We showed in **paper IV** that ART initiation during primary HIV-1 infection (PHI) (early ART=EA) did not prevent the establishment of phenotypical changes of T cells, previously reported in HIV-1 infected patients starting treatment during the chronic phase of infection (late ART=LA). The phenotypical changes of T cells, comparable in the EA and LA groups, consisted in increased expression of immune activation markers HLA-DR and CD38 and reduced expression of CD127, which characterizes differentiated CD8+ T cells. It is worrisome that ART initiation during PHI does not correct for abnormal immune activation. It is however interesting that the number of HIV-1 DNA copies in blood of EA patients was significantly lower compared to LA patients; the correlation between T cell phenotype and size of the HIV-1 reservoir should be studied further. The frequency of B cell sub-populations in blood of EA and LA patients did not differ (**preliminary results**) and was not significantly altered compared to non-infected controls.

CONTENTS

LIST OF ABBREVIATIONS

2-LTR	2-long terminal repeat
AID	Activation-induced cytidine deaminase
AIDS	Acquired immune deficiency syndrome
AM	Activated memory
APC	Antigen-presenting cell
APRIL	A proliferation-inducing ligand
ART	Antiretroviral treatment
BAFF	B cell activating factor
BAFFR	BAFF receptor
BCMA	B cell maturation antigen
BCR	B cell receptor
BM	Bone marrow
CCR	C-C chemokine receptor
CD	Cluster of differentiation
cDNA	Complementary DNA
CFSE	Carboxyfluorescein succinimidyl ester
CM	Central memory
CMV	Cytomegalovirus
CNS	Central nervous system
CTL	Cytotoxic T lymphocyte
CXCR	C-X-C chemokine receptor
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
EM	Effector memory
FCR	Fibroblastic reticular cell
FDC	Follicular dendritic cell
GALT	Gut-associated lymphoid tissue
GC	Germinal center
GI tract	Gastrointestinal tract
gp	Glycoprotein
HIV	Human immunodeficiency virus
HIV-1	Human immunodeficiency virus type 1
HIV-2	Human immunodeficiency virus type 2
HLA	Human leukocyte antigen
ICOS	Inducible costimulator
Ig	Immunoglobulin
IL	Interleukin
IFN	Interferon
INP	Intact-non-induced provirus
LN	Lymph node
LPS	Lipopolysaccharide
LTNP	Long-term non-progressor
MHC	Major histocompatibility complex
MZ	Marginal zone
NHP	Non-human primate
NK	Natural killer

NRTI	Nucleoside reverse transcriptase inhibitor
NNRTI	Non-nucleoside reverse transcriptase inhibitor
PCR	Polymerase chain reaction
PD-1	Programmed death-1
PHI	Primary HIV infection
RM	Resting memory
RNA	Ribonucleic acid
RT	Reverse transcriptase
sCD14	Soluble CD14
SHM	Somatic hyper mutation
SIV	Simian immunodeficiency virus
SLE	Systemic lupus erythematosus
TACI	Transmembrane activator and CAML interactor
TCR	T cell receptor
TD	T cell-dependent
TEMRA	Effector memory RA
Tfh	Follicular T helper cell
TGF	Transforming growth factor
TI	T cell-independent
TLM	Tissue-like memory
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Treg	Regulatory T helper cell
VL	Viral load

1 HUMAN IMMUNODEFICIENCY VIRUS (HIV)

1.1 The discovery of HIV

More than three decades have passed since the first cases of acquired immune deficiency syndrome (AIDS) were reported in New York and San Francisco. At the beginning of the epidemics AIDS was thought to be a disease of homosexual men since previously healthy, young homosexual men were suffering from opportunistic infections and rare types of cancers, symptoms of an impaired immune system [1]. In 1983, two years after the first case was reported, the causative agent of AIDS was isolated [2] and later on named human immunodeficiency virus (HIV). HIV spread rapidly world- wide and cases in heterosexual persons and infants revealed that transmission could also occur through heterosexual contact and from infected pregnant mothers to their children [1]. HIV has of today infected more than 70 million people, and about 34 million people have died of symptoms related to HIV infection [3]. The introduction of combination antiretroviral treatment (ART), approximately 20 years ago, was the beginning of a new era as the new treatment regime could rapidly reduce morbidity and mortality in HIV infected people. Although there has been an improvement in recent years for the access to ART in low-and middle-income countries, far too many people still die of AIDS-related diseases [4] making the HIV epidemic one of our most important global health challenges.

1.2 Epidemiology

The HIV epidemic arose from multiple zoonotic infections of simian immunodeficiency virus (SIV) from African primates to humans in West and Central Africa at the beginning of the 1900s [5]. HIV is a lentivirus for which two types exist, HIV-1 and HIV-2. HIV-1 was transmitted to man from chimpanzees and HIV-2 from sooty mangabey monkeys. HIV-2 is predominantly restricted to West Africa and gives rise to a similar disease as HIV-1, but with a reduced transmission rate and less pathogenicity [5-7]. HIV-1 is distributed into groups M, N, O and P. Group M, which is the main group causing the global HIV pandemic, is further divided into nine subtypes (A-D, F-H, J and K) and many circulating recombinant forms. Subtype C predominates in Africa and India, whereas subtype B is more common in Western Europe, the United States and Australia [7, 8]. As a result of these epidemiological features and of the income of affected countries, most research has been made on HIV-1 subtype B.

In 2014, an estimated 36.9 million people were living with HIV (Figure 1); out of these individuals, around 2 million people were newly infected while 1.2 million people died of AIDS-related diseases [4]. The highest HIV/AIDS morbidity and mortality have been seen in developing countries, with young adults in sub-Saharan Africa experiencing the highest prevalence [5].

As of June 2015, 15.8 million people were on ART globally, 41 % of all adults and 32 % of all children [4]. To take the AIDS response forward, UNAIDS has developed an ambitious approach to reach some targets by 2020. The targets include 90 % of all people being HIV-positive knowing their status, 90 % of people who know their status having access to treatment and 90 % of people on ART being successfully treated with suppressed viral loads (VLs). Of the 36.9 million people living globally with HIV 17.1 million are not aware of their HIV status, while around 22 million do not have access to HIV treatment, including 1.8 million children [9]. A recent report from the European Centre for Disease Prevention and Control concluded that many countries in Europe are still far away from achieving these goals while Sweden is the only country in Europe currently meeting all three targets. The UK is meeting two of these targets but not the one which expects >90% of those estimated to be living with HIV to be diagnosed [10].

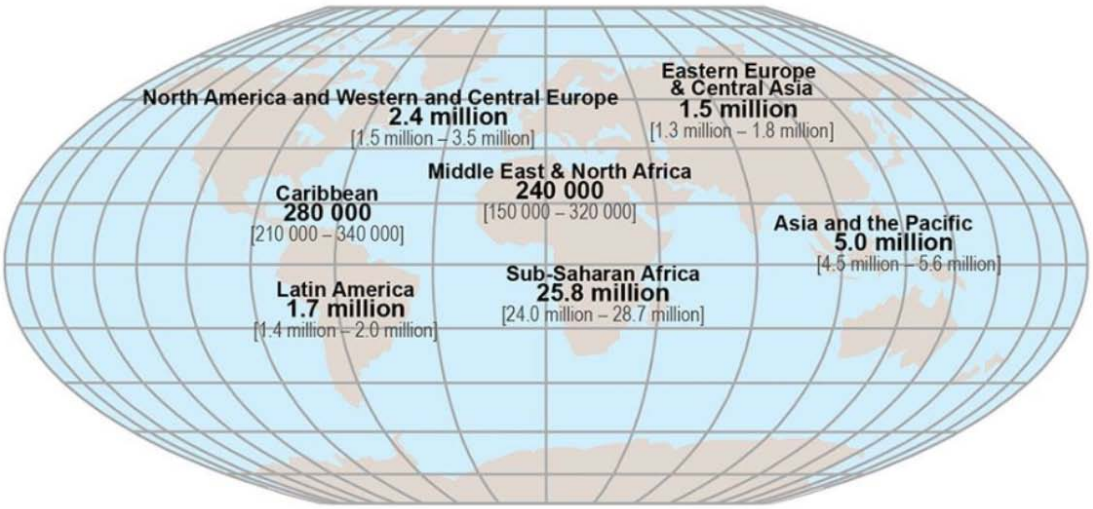

Figure 1. Estimated number of adults and children living with HIV in 2014. Data from UNAIDS 2015 global statistics.

1.3 HIV-1 replication

The HIV-1 replication cycle begins with the interaction of the glycoprotein gp120 on the virus and the CD4 molecule expressed mainly on activated T cells and the chemokine co-receptors CXCR4 or CCR5; X4-tropic HIV-1 strains use CXCR4 and R5-tropic HIV-1 strains use CCR5. Binding of the virus to either of these co-receptors leads to conformational changes of the virus glycoproteins resulting in the fusion of the viral membrane with the membrane of the target host. Other cells bearing the CD4 molecule and the co-receptors are also infected, including resting CD4+ T cells, dendritic cells (DCs), monocytes and macrophages. R5 viruses represent the major group of transmissible strains, whereas X4 viruses tend to arise later on in the course of the disease [11, 12]. In addition to gp120, the HIV-1 envelope complex consists of the transmembrane glycoprotein gp41, which is responsible for the fusion between the viral envelope and the host membrane; after fusion the virus enters the cytoplasm of the host where the viral enzyme called reverse transcriptase (RT) will enable the initiation of virus replication [11].

A double stranded DNA copy of the viral RNA is synthesized by RT followed by the transfer of the viral DNA to the cell nucleus for its integration into the host genome with the help of the viral integrase enzyme. The integrated viral DNA is called provirus [13]. When an extrinsic stimulus activates the infected cell, the cell responds by turning on the transcription of its own genes and by producing cytokines. This process of cellular activation may also activate the provirus that will be transcribed to new viral RNAs and translated into viral proteins that translocate to the cell membrane to become new immature virus particles. The new viruses bud off and are released from the infected cells. In the final step of the HIV-1 replication cycle the virus mature and the protease enzyme cleaves the viral polyprotein giving rise to infectious virions ready to infect other cells [14, 15]. The lack of RT proofreading during the retro-transcription process leads to HIV-1 RNA sequence with a high rate of mutations; this sloppy process of reverse transcription is responsible for virus escape from immune responses and development of drug resistant viruses [5].

1.4 NATURAL COURSE OF HIV-1 INFECTION

1.4.1 HIV-1 transmission

HIV-1 spreads by sexual contact across mucosal surfaces and by percutaneous and perinatal routes. Sexual contact between heterosexual individuals accounts for most transmissions, and HIV-1 is mainly a sexually transmitted disease [5, 16]. Both biological and behavioral factors influence the transmission risk; VL is very high during primary infection and late clinical stages of HIV-1 infection and patients are therefore most infectious during those stages [16]. The presence of sexually transmitted infections, pregnancy and anal intercourse are other factors that increase HIV-1 sexual transmission while male circumcision is associated with a lower risk of transmitting HIV-1 [7]. Although there are tools available to lower the transmission risk close to zero, e.g. the use of condom or initiation of ART in all diagnosed HIV-1 infected patients, UNAIDS has identified discrimination against high risk groups such as iv-drug users, sex workers, men who have sex with men and stigma against HIV-1 infected individuals as the biggest barriers for people to be aware of their HIV-1 status, initiate treatment and access prevention measures [17].

1.4.2 Primary HIV-1 infection

After transmission, the virus disseminates and replicates quickly in lymphoid organs, a period known as the eclipse phase (Figure 2). This phase, which lasts up to 10 days, is when the infection takes place in target cells and organs, but before viral RNA is detectable in the plasma [18, 19]. The burst of viremia is manifested in most patients by an acute HIV-1 syndrome, on average two weeks after primary encounter with the virus. This phase has been denominated as primary HIV-1 infection (PHI). This flu-like illness may last from a few days up to a month and is characterized by a sudden onset of fever, sore throat, skin rash, enlarged lymph nodes sometimes also with headache, night sweats and diarrhea. The symptoms of PHI may be mild enough to pass unnoticed. The viremia peaks at about three to four weeks post exposure [20], and at this time there is a massive depletion of CD4+ T cells, mainly in the gut associated lymphoid tissue (GALT) [21] (Figure 2). Latency is established, within days from initial infection [22, 23], and once the host cell is reactivated by various cytokines, a recall antigen or treatment interruption, latently infected cells are capable of producing infectious virus [24].

Subjects with PHI are maximally contagious due to high viral replication and during this period the risk of transmission is very high [25]. The first weeks following HIV-1 infection can be divided into distinct clinical stages based on viral replication and evolving antibody responses,

the so called Fiebig stages I-VI [26]. After the eclipse phase, HIV-1 RNA can be detected in the blood by polymerase chain reaction (PCR) (Fiebig stage I). Around seven days later tests to detect p24 antigen becomes positive (Fiebig stage II). The p24 antigen is a viral core protein which appears transiently in blood before the development of HIV-1 antibodies; p24 is measured by enzyme-linked immunosorbent assay (ELISA) [25]. Fiebig stage III occurs within approximately five days after the p24 antigen test scores positive and 1-2 weeks after the onset of primary HIV-1 infection; at stage III, HIV-1 antibodies reach levels detectable with sensitive ELISAs.

Fiebig stage IV is characterized by an intermediate Western blot test positivity, where reactivity to two of the following three proteins p24, gp41 and gp120 occurs, approximately three days after the positive results of sensitive ELISA tests. After additional 7 days, or around 1 month after the initial HIV-1 infection, Fiebig stage V occurs, with a clearly positive Western blot test including the three protein bands detected in stage IV; at this stage however, there is still a lack of serum reactivity to polymerase 31 (p31). Stage VI has an open-ended duration, but includes a full Western blot reactivity with a positive p31 band around 100 days after initial HIV-1 infection (Figure 2) [25, 26].

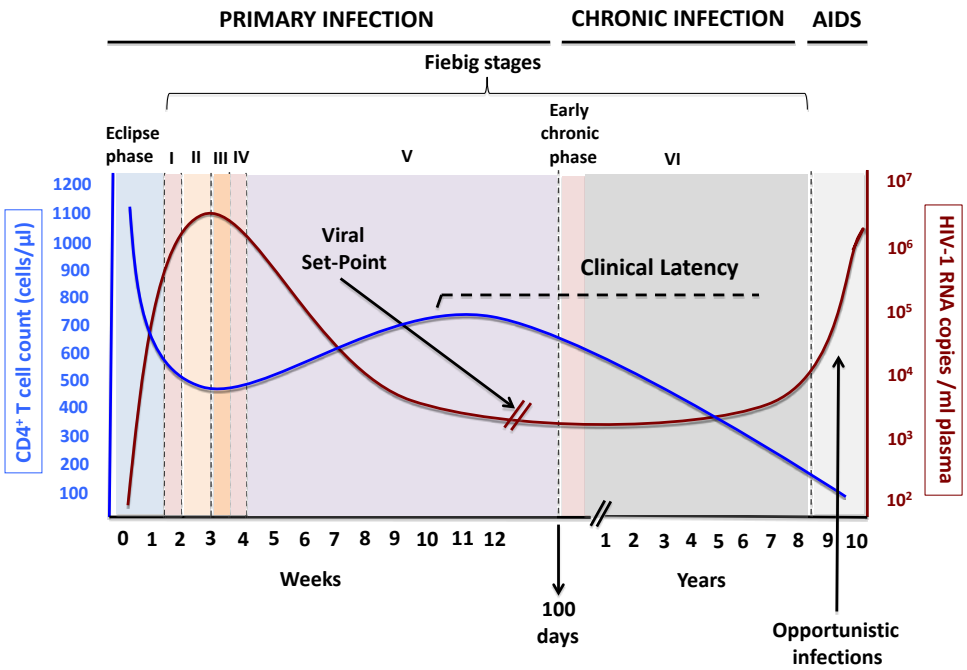

Figure 2. Natural course of HIV-1 infection, adapted from [18].

1.4.3 Chronic HIV-1 infection

Within 3-6 months after infection with HIV-1, plasma viremia will decrease to a stable level, known as the viral-set point (Figure 2). Viral-set point levels are partially predictive of the disease progression rate: the higher the VL is at this stage, the faster disease progression in HIV-1 infected individuals not receiving ART [18, 21]. Host factors, such as different HLA alleles and mutations of the CCR5 receptor, are also playing a role when determining the rate of disease progression [27]. Chronic infection is characterized by a constant, or slowly increasing, level of viremia, whereas the CD4+ T cell count declines slowly in a linear manner. At this point, most patients are asymptomatic and unaware of their infection. This period normally lasts from 1 up to 10 years. Despite the term "latency" the infection is highly dynamic, with abundant CD4+ T cells being infected and killed every day [19].

1.4.4 AIDS

Eventually, as the CD4+ T cells decrease, the immune system deteriorates, the viral "steady state" is lost and viremia increases. When the number of CD4+ T cells declines below 200 cells/μl, HIV-1 infection progresses to AIDS, in general 8-10 years from primary infection. By the time AIDS develops, the CD4+ T cell counts continue to decline and the VL rises further. The infected individual may experience several different opportunistic infections and development of rare cancers, which normally do not develop in HIV-1 non-infected individuals. Without treatment AIDS is soon culminating in death of the infected patient [19].

1.5 Antiretroviral Therapy

The introduction of ART regimens in the late 1990s dramatically increased the life expectancy of millions of HIV-1 infected individuals. HIV-1 infection was transformed from a progressive disease with a deadly outcome into a chronic controlled disease [7]. Antiretroviral drugs are divided into six different groups on the basis of the step of the virus life cycle they inhibit (Figure 3).

Viral entry is the target for various classes of antiretroviral drugs, such as chemokine receptor antagonists that prevent the binding of gp120 to the co-receptor and inhibit entry of CCR5-tropic viruses, and fusion inhibitors, which bind to the surface protein gp41 on the virus. RT is a multifunctional enzyme which transforms the single-stranded HIV-1 viral RNA into double-

stranded DNA; this enzyme is the target for two distinct classes of antiretroviral agents: the nucleoside reverse transcriptase inhibitors (NRTIs) and the non-nucleoside reverse transcriptase inhibitors (NNRTIs). The NRTIs and NNRTIs interact at different sites on the enzyme, which block the DNA polymerization activity and inhibit the generation of full-length viral DNA. Integrase inhibitors specifically inhibit the incorporation of the virus genome into the DNA of the host cell. Protease inhibitors block the proteolysis of the viral polyprotein, needed for generating infectious viral particles. Standard antiretroviral therapy regimens ususally combine two NRTIs with one NNRTI, integrase inhibitor or protease inhibitor.

By combining three or four drugs from different groups long-term viral suppression is maintained and the risk for drug resistance is decreased [28, 29]. CD4+ T cell count and HIV-1 RNA are the main markers used for evaluating the treatment regime. Successful treatment is achieved when the levels of HIV-1 RNA in plasma decrease significantly within four weeks of treatment and to undetectable levels (< 50 copies/ml) within 3-6 months after initiation of treatment. In non-treated HIV-1 infected individuals levels of HIV-1 RNA can give some indication of disease progression, including how fast CD4+ T cells are expected to decrease. In addition, CD4+ T cell count after treatment initiation gives an indication of how compromised the immune system is. According to the latest WHO guidelines, ART should be introduced, independently of the CD4 T cell count, as soon as a patient has been diagnosed with HIV-1 or if there is a clinical suspicion of HIV-1 infection [30]. Increasing data have shown that the introduction of ART in the early asymptomatic phase of the infection is beneficial regarding the prevalence of both serious AIDS-related and non-AIDS related events [31] as well as in slowing down disease progression [32] and decreasing the size of the latent HIV-1 reservoir [22, 33, 34]. HIV-1 transmission is decreased to minimal levels when an infected individual is well treated, further confirming the advantage of initiating ART in the primary phase of infection [30].

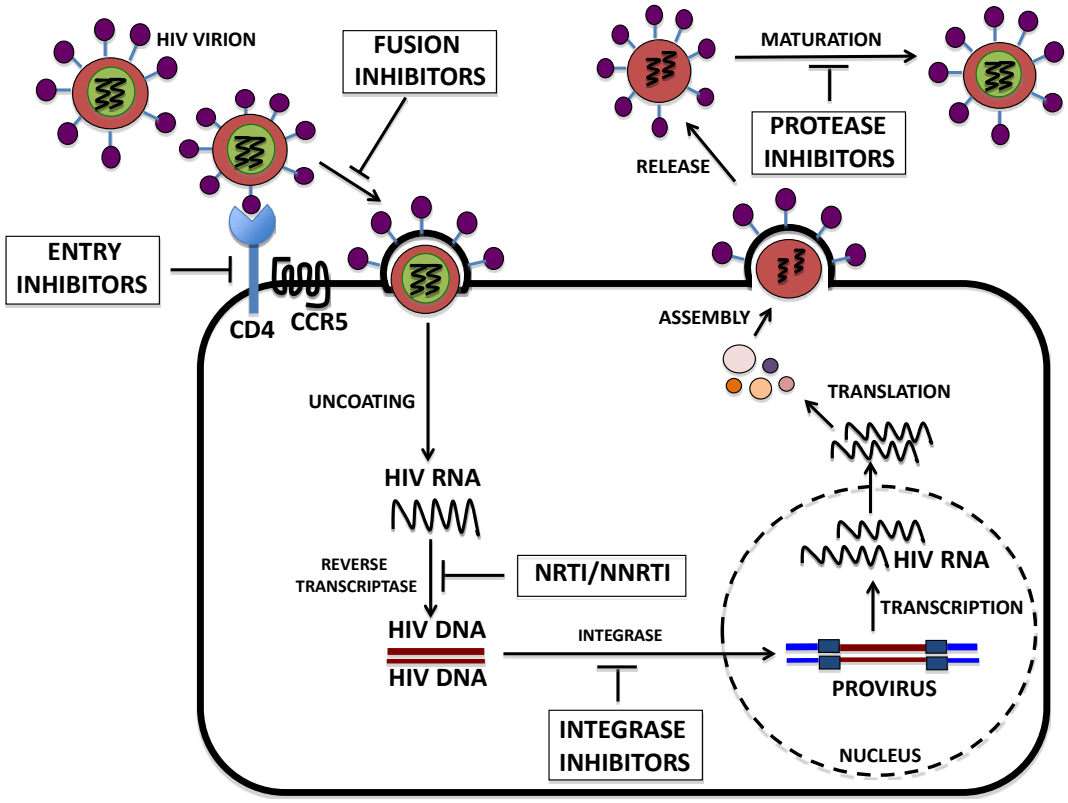

Figure 3. Schematic overview of the HIV-1 replication cycle and the classes of antiretroviral drugs blocking the different steps of virus replication. Adapted from [15].

1.6 Latent HIV-1 reservoir

Although ART is efficient in suppressing VL below levels of detection and restoring CD4+ T cell counts it is not curative as of the persistence of a latent viral reservoir mainly in resting memory CD4+ T cells [35-37]. The reservoir is established during early infection when the virus infects activated CD4+ T cells which then return to a resting memory state [38]. A recent study has shown that latency can also be established directly in resting CD4+ T cells, suggesting that virus infection of both activated and resting CD4+ T cells contribute to virus latency [39]. Resting memory cells are keeping the viral genome in an integrated form that, in its latent form, do not produce viral proteins and peptides, and are for that reason not targeted by the immune system or ART [40]. Once ART is interrupted or during suboptimal treatment these transcriptionally silent, but replication-competent HIV-1 proviruses, are capable of resuming replication which results in rebound of viremia [41]. The latently infected resting memory CD4+ T cell reservoir decays slowly; in fact, the half-life in chronically infected adults has been estimated to 40-44 months, indicating that ART would be required for more than 70 years for its eradication [38].

Different mechanisms contributing to the maintenance of the HIV-1 reservoir during ART have been suggested. There are studies suggesting a continued, low-level viral replication taking place during ART and upon integrase inhibitor intensification active replication is inhibited. In the presence of Raltegravir, which is an integrase inhibitor, integration of linear viral cDNA is blocked and DNA repair enzymes circularize the DNA to form episomes containing two copies of the viral long terminal repeat circles (2-LTR circles) [42, 43]. Another study showed how the stability of the reservoir is maintained via T cell survival through antigen-driven and homeostatic proliferation, suggesting that HIV-1 proviruses in infected cells can expand in the absence of viral replication [44]. Resting CD4+T cells are regarded as the largest reservoir and include different memory CD4+T cell subpopulations, with central memory (T_{CM}), effector memory (T_{EM}) and transitional memory (T_{TM}) CD4+ T cells constituting a significant proportion of the reservoir [45-47]. Other cell types that have been proposed to contain HIV-1 DNA in latent form are macrophages and monocytes [48, 49]. Whether these cells are playing a role in maintaining the HIV-1 reservoir in patients on viral suppressive ART is still unclear.

Studies on the HIV-1 reservoir have primary been performed using peripheral blood; these studies have the limitation that the virus reservoirs in resting memory T cells may mainly be present in lymphoid tissue and not be circulating in blood. The contribution of each subset might also vary when the subsets are sorted from tissues [41]. A number of other anatomical sites and tissue compartments have been proposed to act as reservoirs including the gastrointestinal (GI) tract and central nervous system (CNS). If these anatomical compartments are non-permissive to immune surveillance or have suboptimal drug-penetration viral replication may take place at these sites during ART. GALT is the biggest lymphoid tissue (LT) in the body and studies have shown that it acts as a major tissue reservoir, where memory T cells with persistent HIV-1infection are sequestered in infected individuals on effective ART [50, 51]. HIV-1 DNA has been detected in CNS resident macrophages in individuals on long-term suppressive therapy [52] and in lymph nodes where CD4+T cell populations are circulating. However, the contribution of these compartments to HIV-1 persistence has not yet been fully characterized and should therefore be thoroughly studies for the development of sufficient cure strategies.

The reservoir in resting memory CD4+ T cells consists of a heterogeneous nature of proviruses that can be divided into two groups; induced proviruses and non-induced proviruses. Induced proviruses can release replication-competent virus after one round of T cell activation, whereas non-induced proviruses do not give rise to virions. Most non-induced proviruses are defective and a majority contains different inactivating defects. However, a small fraction of the non-induced proviruses have fully intact genomes, termed intact- non-induced proviruses (INPs),

which can produce virions after additional rounds of cellular activation. Several assays quantify the HIV-1 reservoir, but none measures the true size; culture based assays detect only induced replication-competent proviruses, while PCR-based assays identify all types of proviruses but cannot separate between replication competent proviruses and defective proviruses [40] (Table 1).

The HIV-1 scientific community has recently turned its attention to the possibility of identifying novel targets for curing HIV-1 infection [53]. One possible strategy to eliminate HIV-1 cellular reservoirs is to induce replication of the latent HIV-1 genome; in presence of ART, the virus newly produced from latent reservoirs should be possibly eliminated before it infects new cellular targets. Another strategy is to improve the immunological responses of HIV-1 infected patients with the hope that T-cell mediated immunity, especially CD8+ T cells, may be able to control virus infection once the patients are taken off ART. These are exciting possibilities.

Table 1. Comparison of assays for measuring the HIV-1 latent reservoir. Adapted from [40].

Assay	Detection method	What it measures	What it excludes
Viral outgrowth assay (VOA) [37, 54, 55]	p24 ELISA RT-PCR	Replication-competent virus	Defective proviruses, INPs, 2-LTR circles
qPCR for HIV-1 DNA [56-58]	qPCR	Total proviral DNA	Proviruses with deletions in amplified regions
qPCR for 2-LTR circles [42, 43]	qPCR	2-LTR circles	Integrated proviral DNA
Cell-associated HIV-1 RNA [59]	RT-qPCR	Proviruses induced to make cell associated HIV-1 RNA	Defective proviruses, INPs

2 ADAPTIVE IMMUNITY

The adaptive immune system consists of humoral and cell-mediated immunity. One of the cardinal features of the adaptive immune system includes the generation of immunological memory. B cells mediate the humoral arm and convey their function by producing antibodies, which neutralize and eradicate extracellular microbes and toxins. T lymphocytes provide cell-mediated immunity that eradicates intracellular microbes; T-cell mediated immunity includes T helper cells (Th; CD4+) which activate phagocytes to destroy ingested microbes and activate B lymphocytes to produce antibodies, and cytotoxic T lymphocytes (CTL; CD8+) that kill infected cells harboring microbes in the cytoplasm [56].

2.1 CD4+ and CD8+ T cells

During a primary immune response, antigen-specific naïve T cells migrate to the T cell area of secondary lymphoid organs to scan for antigens presented by DCs. Naïve T cells that encounter the antigen undergo proliferative expansion and differentiate into effector cells. The activated effector T cells assist with the clearance of infection by migrating to the site of infection and orchestrating adaptive immune responses. After the clearance of the infection, the effector T cells will die and a fraction of primed T cells persists into various antigen-specific memory T cell subsets [56]. Memory T cells are divided into different subsets characterized by their phenotype and functional profiles. The main subsets of circulating memory T cells, T_{CM} and T_{EM} cells, can be distinguished by the expression of CCR7, a chemokine receptor homing to secondary lymphoid organs [57]. The T_{CM} subset, which expresses the lymph node homing receptor CCR7, has the capacity to home to secondary lymphoid organs and to proliferate upon activation and an increased ability to survive. T_{EM} cells, which are $CCR7^{neg}$ have direct effector functions after antigen stimulation [58]. After T cell receptor (TCR) triggering or, to a lesser extent, in response to homeostatic cytokines, such as IL-15 or IL-7, T_{CM} cells can develop into T_{EM} cells. [59].

2.2 B cells

Humoral immune responses can be initiated to either non-protein antigens or protein antigens, the latter including T cell-dependent (TD) responses. Naïve B cells in the lymphoid follicles bind to the protein antigen with specific immunoglobulin (Ig) receptors and are activated to migrate out of the follicles. Cognate CD4+ helper T cells, which already have been activated to differentiate into effector cells, interact with these antigen-stimulated B cells at the edges of lymphoid follicles. Helper T cells, through their T cell receptor, recognize peptide antigens

presented by B cells through their MHC class II molecules and activate B cells by expressing co-stimulatory molecules and secreting cytokines. These signals together stimulate high levels of B cell proliferation and differentiation and induce heavy chain isotype switching [60]. The interactions of B and T cells, intensified by signals from follicular dendritic cells (FDCs), leads to the differentiation of T cells into follicular helper T (Tfh) cells [61]. Tfh cells are defined by follicular location and high expression of CXCR5, the T cell inhibitory receptor programmed death 1(PD-1), and production of IL-21 [62]. Tfh cells in turn, drive B cell differentiation further, allowing them to proceed into a germinal center (GC) reaction where affinity maturation and the induction of somatic hyper mutation (SHM), is taking place. GCs are microenvironments that give rise to secondary B cell follicles [63]; GCs are usually formed a few days and up to a week after initiation of an immune response, and persists for weeks or months depending on the antigen (Figure 4) [64].

The ligand from the costimulatory molecule CD40 (CD40L) and the inducible costimulator (ICOS) have been shown to be required for T_{FH} cells and GC development, whereas CXCR5 and the adaptor SLAM associated protein (SAP) are important for T_{FH} cells and GC reaction. In addition, CD40L, IL-21 and IL-4 play major roles in regulating GC B cell proliferation, survival and affinity maturation. B cells can also develop in their absence of these signals but their function is defective [62, 65]. Lack of IL-21R signaling on B cells is associated with lower levels of the transcription factor Bcl-6, resulting in reduced B cell proliferation and switching to IgG1 in GCs [65].

Affinity maturation is giving rise to B cells with high affinity B cell receptors (BCRs) to bind the specific antigen, through a process where the affinity of antibodies for protein antigen increases with prolonged or repeated exposure to the antigens [66]. Heavy chain isotype switching may also occur throughout the GC reaction and not only before the formation of the GC [67]. The exit of B cells from the GC reaction gives rise to both long-lived plasma cells which home to the bone marrow (BM) [68] and memory B cells which are mainly preserved in secondary lymphoid organs with a proportion recirculating in the periphery [64].

Marginal Zone (MZ) B cells belong to a subset of B cells found in the marginal zone of the spleen and act as innate-like lymphocytes able to initiate rapid antibody responses both to T-cell independent (TI) and TD antigens. MZ B cells express less specific BCRs and high levels of toll-like receptors (TLRs) which are binding to microbial molecules such as lipopolysaccharide (LPS) or polysaccharides.

The antigen binding to BCR and TLR molecules activate MZ B cells by having transmembrane activator and CAML interactor (TACI) binding to both a proliferation-inducing ligand (APRIL) and B cell activating factor (BAFF) released by neutrophils, macrophages and DCs. Neutrophils in the spleen release IL-21, which induces the expression of activation-induced cytidine deaminase (AID), class switching to IgA and IgG; antibody-secreting plasmablasts receive additional maturation and survival signals from CXCL10, IL-6 and type I IFNs produced by antigen-capturing cells through a TI-pathway (Figure 4) [69].

Blood-borne bacteria also express TD antigens, like outer-membrane proteins which are processed to peptides and presented to CD4+ T cells through MHC class II. MZ B cells are better antigen-presenting cells (APCs) than follicular B cells, with increased levels of MHC class II, CD80 and CD86 and may participate in the activation of naïve T cells, at least in mice [69, 70]. For GC reaction to take place MZ-primed naïve CD4 T cells need to differentiate into Tfh cells, which thereafter stimulate antigen-specific B cells through a CD40-dependent pathway that leads to the expression of class-switched high affinity antibodies and long-lived memory B cells and plasma cells. A subset of these Tfh cells also interact with extrafollicular MZ B cells and promotes the generation of low-affinity IgM and IgG [69].

It is debated whether human IgD+IgM+CD27+B cells in the blood are generated from GC responses or in the splenic marginal zone independently of T-cell help [71]. Human IgD+IgM+CD27+B cells have been shown to share IgV gene mutations with GC-derived IgG memory B cells; it has been suggested that these cells are able to respond to TD-antigens although they leave the GC reaction before switching to other isotypes [72]. In addition, these cells have also been shown to be present in patients with either CD40 or CD40L deficiency, thus indicating that this subset, can at least in part be generated independently of T cell help [73].

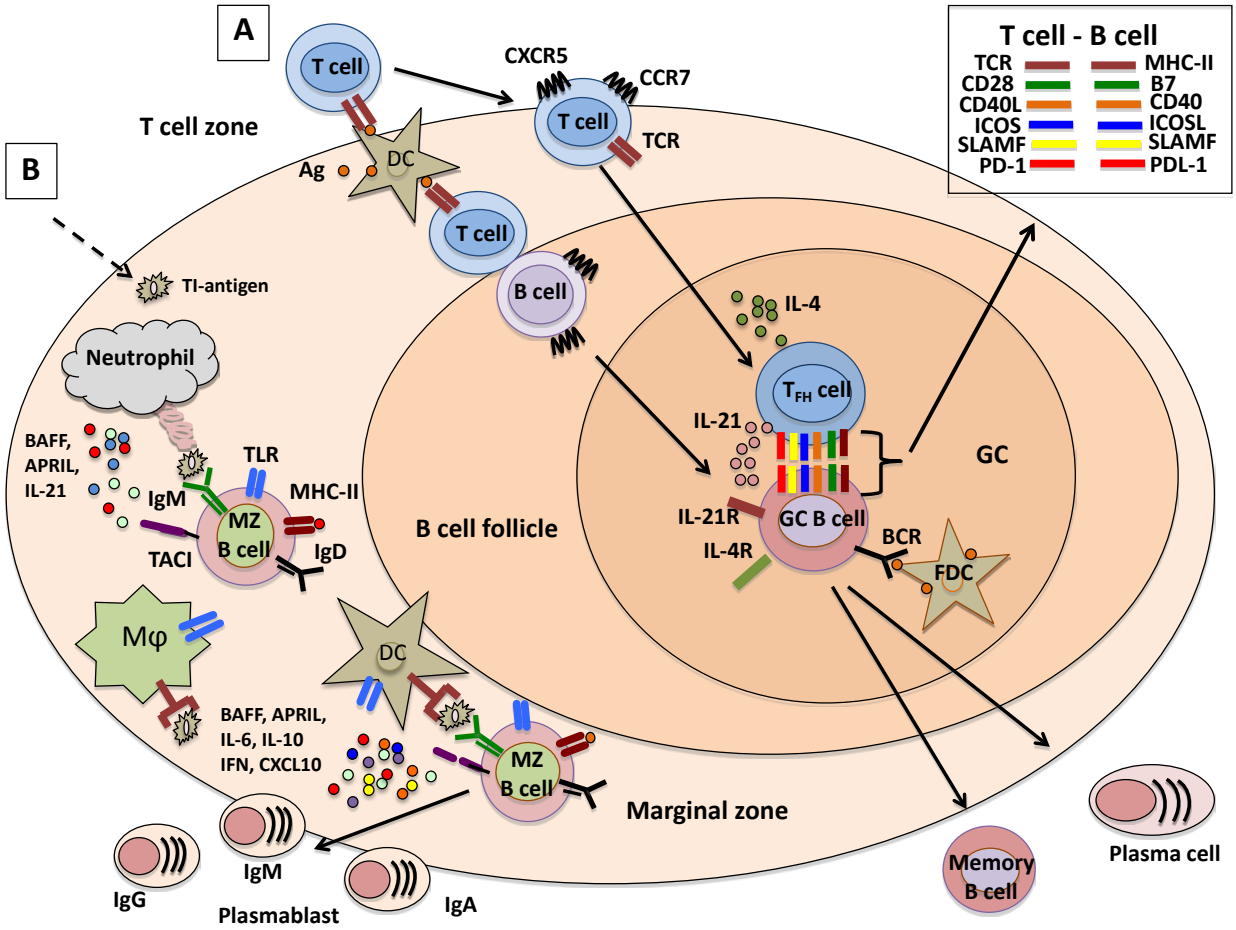

Figure 4. Follicular B cells involved in T cell dependent immune responses involving GC reactions and the production of high affinity antibodies (A) and MZ B cells that rapidly produce low affinity antibodies independently of T cells (B), adapted from [62, 69].

2.2.1 Co-stimulatory pathways regulating B cell responses

Co-stimulatory molecules expressed as receptor and ligand pairs on B cells and T cells are regulating the survival of B cells and T cell-dependent B cell responses; these includes members of the tumor necrosis factor (TNF) and TNF receptor (TNFR) family, such as CD27/CD70 and the CD40/CD40L molecule pairs and BAFF binding to its three different receptors, BAFF receptor (BAFFR), B cell maturation antigen (BCMA) and TACI [74, 75]. The T/B cell crosstalk taking place through CD40L expressed on the surface of activated T cells engaging CD40 expressed by B cells is crucial for T cell dependent humoral immunity and gives rise to the initiation and progression of the GC reaction [76]. CD70 is expressed transiently on T cells, B cells or DCs during activation. The interaction between CD27, expressed on activated B cells

and CD70, when expressed on activated T cells, enhances Ig production by B cells, suggesting that the CD27/CD70 interaction is included in the differentiation of B cells into plasma cells [77, 78]. BAFF is a crucial survival factor for peripheral B cells, and binds to three receptors on B cells; BAFFR is essential for survival and maturation of immature B cells, BCMA promotes plasma-cell survival while TACI is critical for T cell-independent antibody responses, class-switch recombination and as negative regulator of the size of the B cell compartment. BAFF is mainly produced by innate immune cells such as macrophages, neutrophils, monocytes, DCs and FDCs but can also be produced by T cells and B cells [79]. Mice deficient in BAFF lack mature B cells and are immunodeficient [80], while elevated expression of BAFF is associated with autoimmunity; BAFF is in fact a therapeutic target for the treatment of patients with systemic lupus erythematosus (SLE) [81].

2.3 Physiological role of molecules involved in HIV-1 pathogenesis and which will be discussed in the present thesis

HLA-DR is one part of the MHC II molecule, with the other parts being HLA-DP and HLA-DQ. The invariant HLA-DM molecule is involved in loading peptides onto MHC class II molecules while HLA-DO acts as a negative regulator of HLA-DM [82]. The MHC II molecules are highly expressed on APCs such as B cells, macrophages and DCs; through MHC II these cells present processed exogenous antigen to CD4+ T cells and regulate immune responses [83, 84]. The expression of HLA-DR antigen can be up-regulated and down-regulated by different cytokines. T cells express HLA-DR upon activation, although with a slower kinetics when compared to professional APCs [84].

Human CD38 is a surface glycoprotein, initially designated merely as an activation antigen when first discovered in the 1980s. Today we know that this molecule can also behave as a cell surface enzyme (i.e., ectoenzyme) involved in transmembrane signaling, cell adhesion and influencing cell migratory responses [85, 86]. CD38 supports leukocyte trafficking between the blood and the tissues, by controlling the signals that are triggered by chemokine-receptor engagement [101]. CD38 is expressed by immature hematopoietic cells and at high levels by activated B- and T cells and natural killer (NK) cells [85].

CD28 is a co-stimulatory receptor expressed on T cells, involved in the activation of naïve T cells after the engagement of its ligands, B7-1 (CD80) or B7-2 (CD86) [87]. T cell activation includes two signals, first T cell receptor (TCR) recognition and binding to antigen presented on MHC II on the APC. The second signal includes the binding of the B7 ligand on the APC with CD28 on the T cell [88]. The two signals initiate T cell clonal expansion, cytokine secretion and effector functions. Blockade of CD28 signaling results in inefficient T cell activation and CD28 is gradually down-regulated when central memory cells differentiate into effector cells [89].

CD57 is a terminally sulfated glycan carbohydrate. CD57 expression increases with age, from absence in newborns to around 15-20% in adults and is frequently expressed on T cells in individuals with chronic immune activation [90]. CD57 expression on CD8+ T-lymphocytes identifies terminally differentiated cells with reduced proliferative responses to TCR triggering and the cytokines IL-2, IL-7 or IL-15, as well as increased sensitivity to antigen-induced apoptosis [91].

PD-1and its ligands, PD-L1 and PD-L2 regulate the balance between T cell activation, tolerance and immunopathology. PD-1 is an inhibitory receptor expressed on activated T cells, B cells, NK T cells, activated monocytes and DCs. PD-L1 is constantly expressed on macrophages, T and B cells, DCs and BM-derived mast cells and is upregulated on different cell types after activation. PD-L2 is expressed on DCs, macrophages, and BM-derived mast cells [92]. HIV-1 has found a way to use the PD-1: PD-L pathway to avoid immune responses and to maintain persistent infection; functional dysregulation of CD8+ T cells is a reason for an inefficient viral control during HIV-1 infection in humans [93, 94].

The receptor for IL-7 is a heterodimer molecule composed of IL-7Rα and the γ-chain [95]. The α-chain of the IL-7 receptor is also named CD127. IL-7 is needed for T cell maturation, naïve and memory T cell survival and to stimulate T cell activation [96]. In the periphery, both naïve CD4+ and CD8+ T cells express high levels of IL-7Rα and TCR signaling downregulates its expression. T_{CM} and T_{EM} cells both express IL-7Rα, with the highest levels found on T_{CM} cells. [95]. Persistent HIV-1 infection is associated with exhausted CD8+ T cells that express low levels of IL-7Rα and high levels of PD-1 [93], a phenotype which contrasts the CD8+ memory T cells that emerge following the clearance of an acute viral infection, characterized by high levels of expression of IL-7Rα and efficiently maintained for long term without antigen via IL-7 and IL-15 homeostatic self-renewal [97] The expression of IL-7Rα is considered to be a correlative marker of protective antiviral immunity, whereas lack of IL-7Rα expression appears to correlate with failed immunity [95].

CD95, also known as Fas, is a transmembrane protein and a member of the TNF-R family. Its physiological ligand CD95L (FasL) is a member of the TNF cytokine family and together they are part of the extrinsic apoptotic pathway, mediating apoptosis with the main purposes of maintaining T-cell mediated immune responses and deleting autoreactive T cells [98]. CD95 is ubiquitously expressed on most cells, while CD95L is mainly expressed on activated T cells and NK cells [99]. Once the engagement of the death receptor CD95 by CD95L occurs, apoptosis will be triggered; to prevent killing of healthy cells the expression of CD95L is highly regulated [98]. A well established pro-apoptotic activity of CD95 is to mediate the apoptotic death of either cancer cells or virus-infected cells when triggered by CD95L on a CD8+ T cell [99].

3 HIV-1 PATHOGENESIS

3.1 CD4+ T cell depletion

The hallmark of HIV-1 infection is the massive depletion of CD4+ T cells. Although many treated HIV-1 infected individuals are able to fully suppress viral infection, ART fails to regenerate CD4+T cells to pre-infection levels [100, 101]; also low CD4+ T cell nadir has been shown to be a crucial factor in the inadequate immune recovery after ART [102]. In addition, a study was showing that HIV-1 infected patients that maintain an abnormally low CD4+ T cell count despite many years of suppressive ART, had low levels of CD4 + T_{CM} and CD4+ T_{EM} cell populations, with elevated levels of immune activation and high turnover rates, as compared with successful treated HIV-1 infected patients and non-infected subjects [103]. These findings argue for the importance of early ART initiation in HIV-1 infected patients, which would lead to an enhanced recovery of CD4+ T cells and improved long-term immune function in ART treated patients.

CD4 + T cells can be characterized into Th1, Th2, Th17, Tfh and regulatory T helper cell (Treg) subsets based on location, function and cytokine profile (Table 2).

Table 2. Cytokine profile and functions of different subsets of CD4+ T helper cells

	Location	Function	Cytokine profile
Th1	Ubiquitous	Intracellular infection	IFN-γ
Th2	Ubiquitous	Humoral immunity against parasites	IL-4, IL-5, IL-13
Th17	Mucosa	Mucosal immunity against parasites	IL-17, IL-22
Tfh	LTs, GCs	Humoral immunity	IL-21
Treg	Ubiquitous	Suppression of immune responses	TGF-β, IL-10

3.1.1 Alterations in T helper cell subsets

Th17 cells are important for mucosal immunity and their massive depletion in the GALT in the first weeks of HIV-1 infection contributes to the impaired regulation of the epithelium and the breakdown of the mucosal barrier. In HIV-1 infected individuals, Th17 cell depletion correlates with chronic immune activation, microbial translocation, and disease progression [104, 105]. The depletion of CD4+ Th17 cells in the gut results in a skewing of the fraction of CD4+ memory T cell subsets, from a Th17 to a Th1 phenotype [106]. Similar results have been shown in studies conducted in non human primates (NHP), with higher Th17 levels in mucosal tissues of healthy animals. In SIV-infected animals, the levels of Th17 cells decrease in the gut and are never restored, and as the infection progresses CD4+ Th1 cells become the main population [104, 106]. These results suggest that in HIV-1/SIV infection, Th17 regeneration is impaired, causing persistent defects in mucosal immunity.

CD4+ T_{CM} cells are long-lived and self-renewing cells located in LNs and other LTs; these cells have limited effector functions but are able to proliferate in response to antigenic re-stimulation. CD4+ T_{CM} maintain the homeostasis of CD4+ T cells by replacing non-self-renewing, short-lived CD4+ T_{EM} [59, 107]. The infection and depletion of the CD4+ T_{CM} cells are, in comparison with the infection of CD4+ T_{EM} cells, considered to contribute more significantly to the chronic immune activation since T_{CM} are located in the lymph nodes, where immune responses are initiated, and their high levels of infection will therefore translate into high viral replication and activation [108].

Tregs are phenotypically characterized as CD25hiCD127low and positive for the intracellular expression of Forkhead Box p3 (Foxp3) protein; they are regulating the effector activity of other immune cells through the secretion of cytokines like transforming growth factor (TGF)-β and IL-10 [109]. In HIV-1 infected individuals, the decreased levels of Th17 cells is giving rise to an increase in Tregs in the blood and GI tract, which has been shown to correlate with increased levels of activated CD8+T cells and plasma markers of microbial translocation [110]. The impact of Tregs during chronic immune activation is still debated; there are studies showing that decreasing Treg numbers and function will limit immune activation and booster HIV-1 specific immune responses [111, 112]. On the other hand, Tregs can act in a beneficial way, and by increasing their numbers and functions could possibly restrict chronic immune activation. This latter assumption is supported by a study [113], describing that both CD4+ and CD8+ T cell activation correlates with depletion of Treg numbers in HIV-1 infected individuals.

There are studies showing that in viremic HIV-1/SIV infected subjects, the frequency of Tfh cells in LTs is either preserved or increased. The increase of Tfh cells in SIV-infected NHPs was shown to be associated with an increase in activated GC B cells [114, 115]. Interestingly, Tfh cells seem to be infected at a similar frequency than other CD4+ T cell subsets, indicating that the expansion of the Tfh cells is not due to a decreased susceptibility to HIV-1/SIV-infection [115-118]. These results suggests that during chronic HIV-1/SIV-infection, Tfh cells both expand in numbers and are infected at a high frequency contributing to HIV-1/SIV replication and production, but their role in maintenance of chronic immune activation in patients receiving ART still remains to be determined.

3.2 HIV-1 specific CD8+ T cell responses

Following an acute infection, pathogen-specific T cells expand and differentiate into activated effector cells and, once the infection is cleared, develop into memory T cells which rapidly progress with effector functions and re-expand upon encountering the same pathogen [56]. During early HIV-1 infection, the first CD8+ T cell responses arise as viremia approaches its peak. During the time of the peak viremia, there is homogeneity of the founder virus indicating that at that time point no immune-driven selection of escape mutants is occuring. As viremia declines to the viral set point, following the peak of the CD8+ T cell response, a rapid selection of mutations occurs in the virus genome [18]. As the infection progresses, the virus evades the CTL responses; this event contributes to the development of a chronic infection. During the infection, HIV-1 specific CD8+ T cells persists and become dysfunctional, a process commonly known as CD8+ T cell exhaustion [93, 119, 120].

3.2.1 T cell exhaustion

Chronic infection, involving persistent exposure to antigen and inflammation, alters the memory T cell differentiation programme. Other factors contributing to T cell exhaustion are increased expression of inhibitory receptors and lack of CD4 + T cell help [97]. Abnormalities linked to exhausted virus-specific CD8 T cells include the loss of IL-2 production and the high capacity to proliferate, followed by the loss of ability to produce TNF-α. As a consequence of severe CD8+ T cell exhaustion, HIV-1 specific cells partially or completely lose their ability to degranulate and to produce IFN-γ and a deletion of virus-specific T cells occurs [119]. During HIV-1 infection, PD-1 is playing an important role in T cell exhaustion; an increased expression of PD-1 on HIV-specific CD4+ and CD8+ T cells correlates with VL, lower CD4+ T cell count and the reduced capacity of CD8+ T cells to proliferate in response to HIV antigen *in vitro* [92, 93, 121].

By comparing PD-1 expression on HIV-1 specific CD8+ T cells in long-term non-progressors (LTNPs) with typical progressors, it was shown that LTNPs express low levels of PD-1 on their HIV-1 specific CD8+ T cells; in contrast, the typical progressors presented with upregulated PD-1 expression on CD8+ T cells which correlated with elevated VL, reduced CD4+ T cell number and decreased HIV-1 specific CD8+ T_{EM} cells [92, 122].

In addition to PD-1, other surface inhibitory molecules have been shown to be expressed on exhausted T cells and the severity of T cell exhaustion has been suggested to depend on the amount of inhibitory receptors expressed in one cell at the same time. There is a debate ongoing on whether the individual expression of PD-1 or other inhibitory receptors is a marker for

exhaustion, or if co-expression of multiple inhibitory receptors is a key feature of exhaustion [119]. Indeed, the high expression of many inhibitory receptors on CD8+T cells is associated with T cell exhaustion in HIV-1 infected individuals [123].

The maintenance of exhausted T cells is also dependent on their transcription factors, such as Eomesedormin (Eomes) and T-box transcription factors (T-bet) and with their different levels of expression helping maintaining the pool of exhausted CD8+ T cells during chronic viral infection [124]. A recent study characterized the levels of EOMES and T-bet in CD8+ T cells from chronically HIV-1 infected, ART treated patients and found that HIV-1 specific CD8+ T cells expressed elevated levels of Eomes and low levels of T-bet, a profile associated with increased expression of inhibitory receptors, dysfunctional features and a transitional memory phenotype; this impaired profile was not normalized despite viral suppression with ART [120].

3.2.2 T cell senescence

T cell senescence is characterized by an expanded population of terminally differentiated CD8+ T cells with shortened telomeres, increased expression of CD57 and down-regulation of the co-stimulatory molecule CD28 [89, 125]. A recent study [126], showed increased levels of immune activation, measured by the increased soluble CD14 (sCD14) levels in plasma and higher percentages of $CD38^+$ HLA-DR^+ $CD4^+$ and $CD8^+$ T cells, in HIV-1 infected ART treated patients compared with controls; the $CD4^+$ T-cell activation in the treated HIV-1 infected patients was inversely associated with $CD4^+$ T-cell count and $CD4^+$ T-cell recovery. Interestingly, increased immune activation was also associated with shorter telomeres, suggesting that chronic inflammation associated with HIV-1 disease drives excess activation and proliferation of T cells, which in turn leads to telomere shortening and ultimately to poor immune recovery and the immune-senescent phenotype. Another study found that the immunosenescent phenotype of CD28- CD8+T cells was evident already within the first few months of infection and reversed by early initiation of ART, but not when ART was delayed by a few years, illustrating the relevance of early initiation of ART [127]. However, our group showed earlier that only CD28- T cells from non-treated HIV-1 infected patients were associated with an immunosenescent phenotype while CD28- T cells isolated from ART-treated HIV-1 infected patients, were more prone to proliferation as compared to cells from non-treated HIV-1 infected and non-infected individuals [128].

3.3 Chronic immune activation

The driving force for CD4+ T cell depletion and the development of AIDS is the systemic chronic immune activation taking place in HIV-1 infected individuals [129]. Although ART can almost completely suppress HIV-1 replication, prevent AIDS and reduce mortality, antiviral drugs are not completely targeting immune activation and several years of viral suppression, residual immune activation persists [129-131]. New recent findings indicate that the suppression of viral replication by ART is incomplete in almost all treated patients [43, 132], suggesting that the levels of activated CD4+ T cells in patients receiving ART may contribute to HIV-1 persistence by constantly providing a pool of cellular targets for virus infection [133].

Treated HIV-1 infected adults, as compared to age-matched uninfected individuals, are at a higher risk of developing non-AIDS related diseases, including cancer, liver, kidney, cardiovascular, neurologic and bone diseases [31, 134]**.** Immune activation, chronic immune dysfunction and inflammation most probably contribute to the increased risk of morbidity and mortality in HIV-1 infected individuals. A few factors have been shown to contribute to the immune dysfunction and persistent inflammation in HIV-1 infected individuals undergoing treatment; (I) untreated HIV-1 infection gives rise to collagen deposition in lymphoid organs, causing irreversible tissue fibrosis, which is not normalized once treatment is initiated and contributes to failed T-cell homeostasis [135, 136]; (II) destruction of mucosal surfaces within the gut, giving rise to microbial translocation [137]; (III) increased replication of common pathogens such as cytomegalovirus (CMV) [138] and (IV) persistent inflammation [133] (Figure 5).

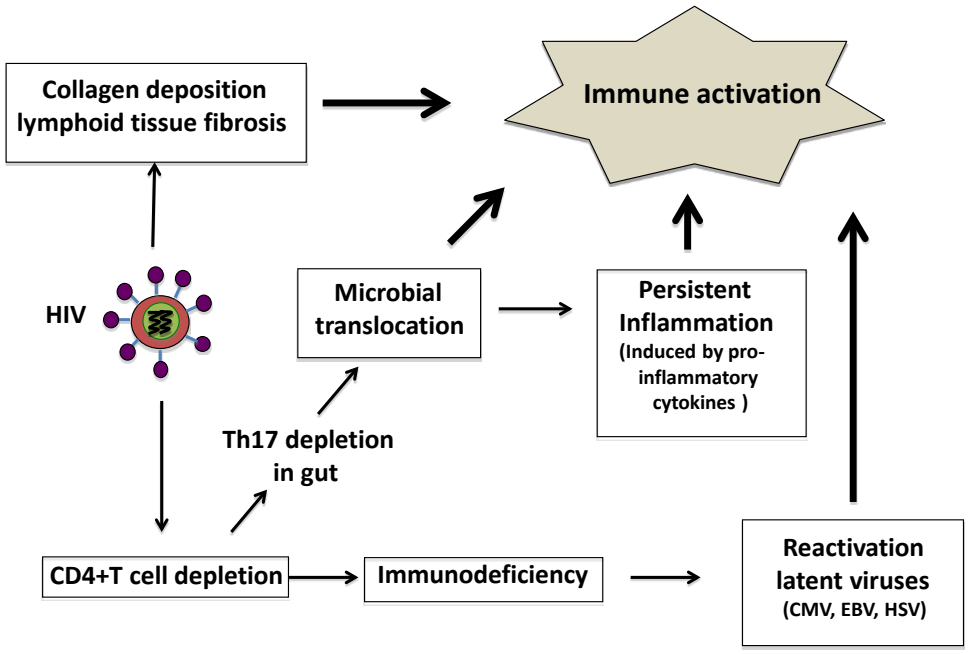

Figure 5. Factors contributing to chronic immune activation in ART- treated HIV-1 infected individuals. Adopted from [129].

3.3.1 HIV-1 induced collagen deposition in secondary LTs

HIV-1 replication occurs mainly in the secondary LTs and as the infection progresses, the high endothelial venules (HEVs) become thickened and sclerotic, giving rise to accumulated collagen throughout the T cell zone. Tregs produce transforming growth factor (TGF)-β1, which stimulate fibroblasts to produce and deposit collagen during the course of infection. The deposited collagen damages the fibroblastic reticular cell (FRC) network, leading to a reduced availability of IL-7 which results in loss of naïve T cells and an increased apoptosis in both CD8+ and CD4+ T cell subsets. For maintaining the FRC network, it physically needs to interact with T cells to receive lymphotoxin signals, which are produced by CD4+ T cells. The FCR network loss is mainly caused by the CD4+ T cell depletion in parallel with collagen deposition leading to the loss of interaction between CD4+ T cells and the FRC network This pathological mechanism is initiated in the early infection, and therefore, initiating ART during PHI correlates with improved preservation of CD4+ T cells and lower apoptosis of naïve T cells in LTs [136].

3.3.2 Microbial translocation

Th17 cells are massively depleted in GI tract within the first weeks of infection; contributing to the disruption of tight junctions, loss of intestinal epithelial cells and weakened mucosal intestinal barrier. The compromised mucosal immunity in the gut is favoring translocation of bacterial and fungal products, such as flagellin, LPS, peptidoglycan and ribosomal DNA, from the lumen to the systemic circulation. These microbial products, through the stimulation of several TLRs, generate different pro-inflammatory cytokines, such as IL-6, IL-1β,TNF-α and type 1 Interferons in different immune cells contributing to the aberrant immune responses during chronic HIV-1 infection [133].

Increased LPS levels in circulation have been described in relation to microbial translocation during chronic HIV-1 infection [139]. LPS makes up the layer of the outer membranes of most gram-negative bacteria and works as an activator of the innate immune system through TLR4 [140]. In studies of Sooty mangabey monkeys that represent natural hosts for SIV-infection and do not develop HIV-1 associated disease, immune activation is not occurring and low levels of LPS are found in plasma and in the colon [137, 141]. In another study, designed to decrease microbial translocation, acutely SIV-infected macaques were treated with an antibiotic and an anti-inflammatory drug; the treatment reduced LPS levels in plasma, and transiently decreased the levels of immune activation and viral replication in treated SIV-infected macaques as compared with non-treated SIV-infected animals [142].

3.3.3 Reactivated infections which may increase immune activation

HIV-1infected individuals are more often present with other chronic viral infections; different latent viruses, such as herpex simplex virus (HSV), cytomegalovirus (CMV) and Epstein-Barr virus (EBV) are due to CD4+ T cell depletion more often reactivated in HIV-1 infected individuals [129]. A few studies have pointed out a role for opportunistic viruses in maintaining chronic immune activation during HIV-1 infection; the activation marker CD38 was shown to be upregulated on CD8+ T cells specific for, EBV, CMV and influenza virus, as well as on HIV-1 specific CD8+ T cells during primary HIV-1 infection [143]. A recent clinical study showed that treatment of HIV-1 infected individuals with an antiviral drug used to treat CMV infection, significantly decresed the levels of HLA-DR+CD38+ CD8+ T cells in ART treated HIV-1 infected individuals with low CD4+ T cell count, further proving evidence that CMV (or/ and other herpesvirus) replication may affect immune activation during HIV-1 infection [138].

3.3.4 Markers of immune activation during HIV-1 infection

HIV-1 infected individuals are usually observed with increased levels of the activation markers HLA-DR and CD38 and Ki-67, the marker for proliferation on both CD4+ and CD8+ T cells [144]. Immune activation is also characterized through the measurement of plasma biomarkers of inflammation, such as C-reactive protein (CRP), TNF-α, IFN-γ, IL-6 , β2-microglobulin (β2M) and the coagulation cascade marker, D-dimer, which are all increased in chronic HIV-1 infection [145-147]. In chronic HIV-1 infection, the levels of the anti-inflammatory cytokines TGF-β and IL-10 are elevated but also predictable of progression to AIDS [148]. β2M is a component of the MHC I complex, released by activated T cells and elevated during HIV-1 infection, which levels inversely correlated with CD4+ T cell counts [146]. In the acute phase of infection, levels of the marker IFN-inducible protein-10 (IP-10) in plasma have been shown to be predictive of rapid disease progression [149] and sCD14, the marker of monocyte activation released in response to LPS and an indirect marker of microbial translocation has been shown to negatively correlate to CD4 T cell counts in treated HIV-1 infected individuals [126].

3.4 B cell dysfunction during HIV-1 infection

HIV-1 infection is in addition to the depletion and dysfunction of CD4+ T cells also associated with activation and dysregulation of B cells. In the early course of infection, HIV-1 replication is driving the immune activation leading to bystander pathological effects on B cells [150]. B cell alterations include loss of serological memory to antigens previously encountered through vaccination and natural infection [151-155].

3.4.1 Direct and indirect effects of HIV-1 viremia on B cells

It has not been shown that HIV-1 can efficiently replicate in B cells as these cells lack the expression of the CD4 molecule, the main receptor for HIV-1. Studies have however showed that there is a direct interplay between the virus and B cells *in vivo*, through the interaction of HIV-1 virions bound to complement proteins and CD21, the complement receptor expressed on mature B cells [156, 157]. In addition to CD21, other HIV-1 binding receptors have been described on B cells, such as DC-SIGN and other C-type lectin receptors, but their role as HIV-1 receptors *in vivo* is unknown [158, 159]. A recent study identified gp120 binding to human B cells via integrin α4β7, contributing directly to B cell dysfunction by activating inhibitory pathways resulting in the increased expression of the immunosuppressive cytokine TGF-β1 and the inhibitory receptor FcRL4 [160]. The frequency of B cells with HIV-1 virions on their

surface is relatively low in comparison to the degree of B cell dysfunctions taking place in HIV-1 viremic individuals, indicating that B cell damage is occurring through indirect mechanisms during HIV-1 infection.

HIV-1 induced aberrant immune activation is giving rise to B cell hyperactivation and hypergammaglobulinemia, the latter phenomenon characterized by increased levels of immunoglobulin in the circulation. Other features of hyperactivation include increased B cell turnover and increased B cell malignancy [151, 161, 162]. The HIV-1 protein Nef appears to affect B cells in multiple ways; infected macrophages that express Nef generated the protein ferritin, which induced B cell activation in HIV-1 infected individuals and IgG levels in the serum correlated with ferritin and VL [163]. In addition, Nef has also been shown to compile on B cells and to prevent Ig class-switch DNA recombination by blocking CD40 ligand signaling pathway [164].

3.4.2 Alterations in B-cell subsets

The B cell abnormalities induced by HIV-1 are not the result of the appearance of new B cell subsets absent in healthy individuals, but rather originate from an imbalanced frequency of B cell subsets present in healthy individuals [150]. An increased frequency of immature transitional (CD19+ CD10+) B cells are present in the blood of HIV-1 infected individuals [165, 166], results from the increased levels of transitional B cells (CD19+CD10+CD21+CD27-) and GC founder B cells (CD19+CD10+ CD21+CD27+) [167]. Transitional B cells respond weakly to BCR stimulation and are more sensitive to spontaneous apoptosis [165]. The pathogenic mechanisms that give rise to the increased levels of transitional B cells in the blood during HIV-1 infection are not fully known but the cytokine IL-7, which is increased during untreated HIV-1 infection, has been shown to influence the ratio of transitional B cells; in fact, the increased levels of immature/transitional B cells are more evident in advanced HIV-1 disease and correlate with increased IL-7 levels [165, 168].

In HIV-1 infected individuals, the frequency of resting memory (RM) B cells (CD19+ CD10-CD27+CD21+) is decreased [153, 161, 169], and correlates with the reduced levels of antibodies against different vaccination antigens (tetanus, pneumococcus and measles) [153, 170]; the decreased maintenance of serological memory has been shown to take place early during HIV-1 infection [171].

A common pathogenic feature for B cells from viremic HIV-1 infected individuals is the over-representation of memory B cells that express low levels of CD21, namely activated memory (AM) B cells (CD19+ CD10-CD27+CD21-), which express the highest levels of activation markers CD80 and CD95 and respond weakly to different B cell stimuli among all B cell subsets [151], and tissue-like memory (TLM) B cells (CD19+CD10-CD27-CD21-) which express the inhibitory receptor FcRL4 and display an exhausted phenotype, including lower epitope diversity, lower capacity to proliferate and a reduced number of cell divisions [172]. Silencing of the inhibitory receptor FcRL4 and Siglec-6 on B cells resulted in increased B cell proliferation and cytokine release in viremic HIV-1 infected patients, confirming the presence of B cell exhaustion during HIV-1 infection [173]. The characteristic exhausted phenotype of TLM B cells include the expression of PD-1, the inhibitory receptor associated with exhausted T cells during HIV-1 infection [93]. In a recent study, PD-1 expression was measured on all memory B cell subsets of HIV-1 viremic individuals, and it was shown that PD-1 expression was elevated only on RM B cells; suggesting that increased expression of PD-1 might be associated with the reduced number of this subset of B cells in HIV-1 infected individuals [174].

The decreased survival of memory B cells in HIV-1 infection has been suggested to be affected by the death receptor-mediated pathway; in fact, viremic and lymphopenic patients has been shown to upregulate CD95 on their memory B cells as compared to healthy controls [174]. In addition, the increased frequency of AM B cells sensitive to CD95-mediated apoptosis correlated with viremia [175].

Table 3. Identification of memory T and B cells in the thesis is based on expression of CD45RA and CCR7 on CD3+CD4+/CD8+ T cells and CD27 and CD21 on CD19+CD10- B cells.

	Naive T cells	Central memory T cells (T_{CM})	Effector memory T cells (T_{EM})	CD45RA+ effector memory T cells (T_{EMRA})	Naive B cells	Resting memory B cells (RM)	Activated memory B cells (AM)	Tissue-like memory B cells (TLM)
CD45RA	+	-	-	+				
CCR7	+	+	-	-				
CD21					+	+	-	-
CD27					-	+	+	-

3.4.3 Reduction of HIV-1 viremia by ART leads to normalization of some B cell dysfunction

During ART, normalization of the frequency of some B cell subsets has been shown to be slow and incomplete. After one year of treatment, the frequencies of AM and TLM B cells had not normalized, suggesting that despite suppression of viremia induced by ART, some immune-activating effects of HIV-1 persist [167, 176]. Hypergammaglobulinemia, which is maintained at elevated levels during chronic viremia is not completely normalized after ART initiation [153, 161]. In addition, HIV-1 infected individuals initiating ART in the chronic phase of infection are not able to restore the RM B cell compartment [152, 176]. However, initiating ART during PHI is associated with improved B cell memory function as measured by improved B cell memory response to a recall antigen in both adults and children [167, 170].

4 AIMS OF THE THESIS

The specific aims of this thesis are:

- Study the impact of microbial translocation and immune activation on dysfunctions of B cells in HIV-1 infected patients (**paper I**).

- Investigate the role of IL-7 on B cell survival and activation, and antibody production (**paper II**).

- Study the role of altered CD70 expression on T cells to B cell abnormalities during HIV-1 infection (**paper III**).

- Investigate whether ART initiated during primary HIV-1 infection prevents the establishment of abnormal phenotypic features of CD4+ and CD8+ T cells (**paper IV**).

- Investigate whether ART initiated during primary HIV-1 infection leads to a preserved B cell compartment (**preliminary results**).

5 RESULTS AND DISCUSSION

5.1 PAPER I - Impact of microbial translocation and immune activation on B cell activation and loss of memory B cells in HIV-1 infected patients

Memory B cells are, in addition to plasma cells, the cellular component responsible for the maintenance of serological response to antigens previously encountered in life through natural infection and vaccination. In the context of HIV-1 infection, different B cell alterations occur including a decline in the frequency of resting memory B cells in both adults and children and loss of serological memory [152, 154] .

Microbial translocation and abnormal immune activation were shown to be involved in impairment of T cell function and disease progression during HIV-1 infection [137]; however, as T cells, also B cells present with activation and exhaustion features [151, 172]. Therefore, we were interested in studying the impact of HIV-1 replication and microbial translocation on frequency of B cells and loss of memory B cells.

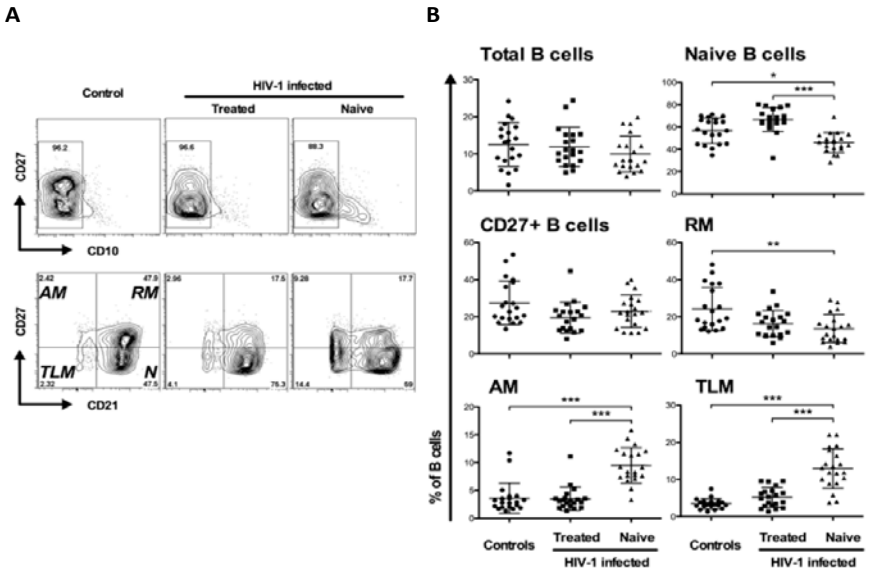

Figure 6. B cell phenotype during HIV-1 infection. Representative plots on the gating strategy of B cell subpopulations gated on live CD19+CD10- B cells (**A**). Frequency of total B cells (CD19+), naïve B cells (CD19+CD10-CD27-CD21+), classical memory B cells (CD19+CD10-CD27+), resting memory (RM) B cells (CD19+CD10-CD27+CD21+), activated memory (AM) B cells (CD19+CD10-CD27+CD21-) and tissue-like memory (TLM) B cells (CD19+CD10-CD27-CD21-) (**B**).

The frequency of B cell sub-populations during HIV-1 infection is shown in Figure 6. As compared to non-infected individuals, untreated HIV-1 infected patients exhibited a decrease of naïve B cells, whereas transitional B cells (CD19+CD10+) were expanded as shown in Figure 6A. As anticipated, the frequency of resting memory (RM) (CD19+CD10-CD27+CD21+) B cells was decreased in HIV-1 infected individuals while activated AM (CD19+CD10-CD27+CD21-) and TLM (CD19+CD10-CD27-CD21-) B cells were found at higher frequency. These results confirm previous studies which found a larger frequency of activated and exhausted B cell cells in the circulation of viremic HIV-1 infected individuals [151, 172]. Following initiation of ART, these alterations are reversed except for the decreased population of RM B cells which is also found in ART treated patients. The abnormal distribution of B cell subsets which we found in HIV-1 infected patients has also been reported in other studies [172, 177].

IL-21R, the receptor for IL-21, has been shown to be upregulated upon B cell activation and to play a role in B cell differentiation and survival [178]; we therefore measured the IL-21R expression on B cells from control subjects and patients (Figure 7A). In general, both in uninfected controls and HIV-1 infected individual, IL-21R expression is high on naïve B cells, AM and TLM cells, the latter finding consistent with their activation status; on the other hand, classical memory and RM B cells display a moderate expression of this receptor. An increased level of IL-21R expression was found on classical memory (CD19+CD10+CD27+) B cells and TLM B cells of viremic HIV-1 infected patients.

We also measured the expression of CD38, a known marker of immune activation. Higher levels of CD38 expression were shown on total B and classical memory B cells from viremic patients, as compared to ART-treated and uninfected individuals. In paper I, CD38 expression on all B cell subsets was inversely correlated with CD4+ T cell counts and correlated with HIV-1 VL for all subsets, except RM and TLM B cells. These results suggest a role for HIV-1 replication, and possibly lymphopenia, on the upregulation of CD38 on B cells from viremic patients (results shown as supplementary Figure 3 in Paper I). No correlation was however found between IL-21R expression on B cells and CD4+ T cell counts or HIV-1 VL.

We further explored the association of IL-21R expression with loss of memory B cells during HIV-1 infection (Figure 7B). We found an inverse correlation between the levels of IL-21R expression in both classical memory B cells and RM B cells and the frequency of these cells in circulation of treated and untreated HIV-1 infected individuals. Of note, no correlation was

found between the CD38 expression on memory B cells and the levels of these cells in circulation for any of the cohorts. Moreover, IL-21R+ memory B cells were more susceptible to apoptosis than memory B cells lacking the receptor, as shown by the lower expression of the anti-apoptotic molecule Bcl-2 and higher expression of Annexin V.

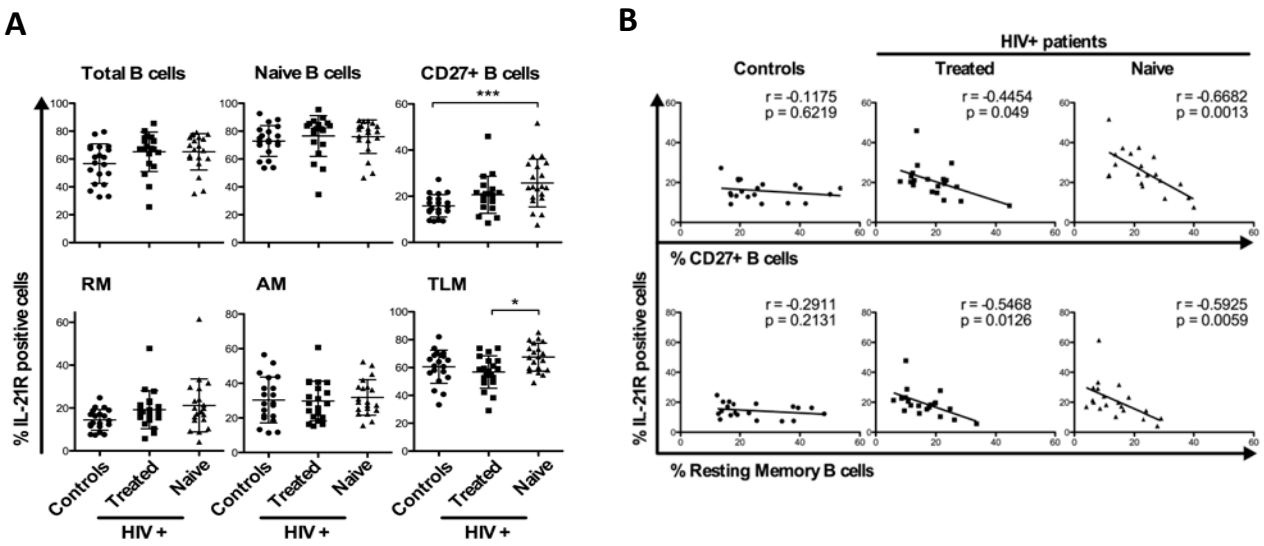

Figure 7. IL-21R expression on memory B cell subpopulations and its correlation with reduced percentages of memory B cells during HIV-1 infection. Frequency of IL-21R positive cells among total, naïve, classical memory, resting memory (RM), activated memory (AM) and tissue-like memory (TLM) B cells (**A**). Correlation of IL-21R expression with CD27+ classical memory (upper panel) and RM B cells (lower panel) in control subjects and HIV-1 infected patients (**B**).

To determine whether microbial translocation could play a role in the establishment of B cell defects during HIV-1 infection, we measured the plasma levels of sCD14 (Figure 8A). The sCD14 levels were higher in HIV-1 infected patients, both viremic and treated, as compared with uninfected controls. To evaluate the impact of immune activation mediated by products of microbial translocation, in modulating IL-21R and CD38 expression on B cells, we correlated the levels of sCD14 with the frequency of different B cell memory subsets and expression of activation markers on these cells. A positive correlation was found between the sCD14 levels and the percentage of AM B cells whereas an inversely correlation was found between the sCD14 levels and RM B cells (Figure 8B). These results suggest that microbial translocation may have a role in the expansion of AM B cells during HIV-1 infection, whereas microbial translocation has a negative impact on the frequency of RM B cells. The expression of IL-21R in

classical and RM B cells correlated with sCD14 levels, whereas CD38 expression in classical and AM B cells correlated with sCD14 levels.

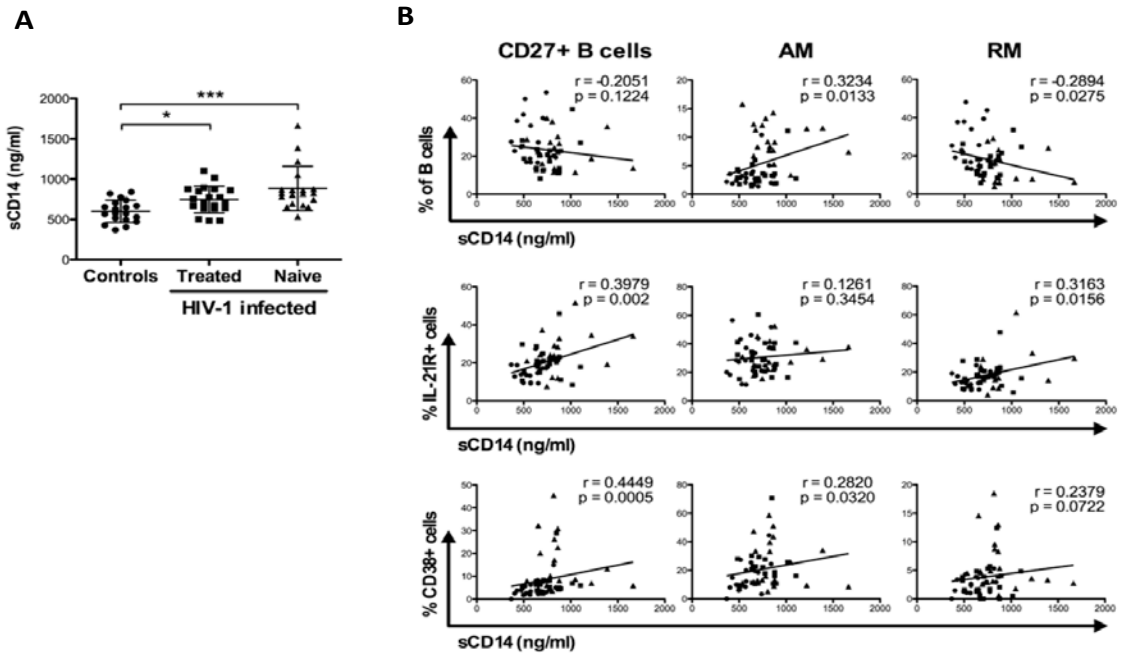

Figure 8. Plasma sCD14 correlates with both activation and loss of memory B cells. Levels of sCD14 were measured by ELISA in plasma samples from uninfected control subjects and HIV-1 infected patients (**A**). The percentages of circulating classical memory (CD27+) B cells, activated memory (AM) and resting memory (RM) B cells and their respective levels of IL-21R and CD38 expression were correlated with the plasma levels of sCD14. These correlations were made including all groups (**B**).

We also aimed at clarifying whether products of microbial translocation have a direct effect on IL-21 and CD38 expression on B cells. For this purpose we incubated separated B cells or PBMCs with different TLR ligands. Purified B cells or B cells in peripheral blood mononuclear cells (PBMCs) up-regulated IL-21R on both naïve and memory B cell subsets when stimulated with TLR-9 and TLR-2 ligands and, to a lesser extent, with TLR-4 ligands. CD38 expression remained unchanged when purified B cells were triggered by TLR ligands; however, CD38 was up-regulated on B cells in PBMC cultures in presence of TLR-3, TLR-4, TLR-7/8 and TLR- 9 ligands. These results reveal a direct effect of TLR triggering on increased IL-21R expression on B cells, but not on CD38 expression, the latter depending on other cellular components present in PBMCs.

Abnormal immune activation is a well-studied feature of T cell immunopathology during HIV-1 infection, which has been characterized through a variety of different surface markers. Very few studies have previously addressed the modulation of IL-21R and CD38 expression on B cells during HIV-1 infection. Chong and co-authors also reported that an elevated expression of CD38 on B cells can be found during untreated HIV-1 infection as a result of viremia [179]. In **paper III** we also studied the expression of CD38 on B cells in different cohorts of HIV-1 infected patients, including patients presenting with viremia and lymphopenia. CD38 expression on B cells was increased in treated patients who maintained a low CD4+ T cell count (< 200/μl) in spite of ART and non-treated viremic patients, as compared to ART treated patients with CD4+ T cell counts >200/μl and healthy controls.

Microbial translocation, resulting from the damaged epithelium barrier in the gut of HIV-1 infected patients, has also been, in many contexts, associated with T cell decline and impairment of T cell phenotypes and function. This has become a central accepted knowledge in the field of HIV-1 pathogenesis. In **paper I** of my thesis it was shown that microbial translocation may also play a role in impairment of the B cell phenotype described to occur in HIV-1 infected patients. Increased levels of sCD14, is associated with both high IL-21R expression on memory B cells, which are more susceptible to apoptosis, and with reduced frequency of these cells in HIV-1 infected individuals.

It would be important to define a panel of markers defining abnormal activation of B cells during HIV-1 infection as these markers may be proven useful to define immune reconstitution and preservation upon early ART intervention.

5.2 PAPER II - Role of IL-7 on B cell activation and survival

Prior to ART introduction, increased levels of IL-7 in serum have often been associated with CD4+ T cell depletion in HIV-1 infected individuals [168]; this finding suggested that T cell depletion leads to high IL-7 levels, which, in turn, may lead to T cell regeneration through increased survival and proliferation. In clinical studies in HIV-1 infected patients, IL-7 has been shown to positively affect T cell biology during HIV-1 infection, by enhancing the survival and expansion of T cells in HIV-1 infected individuals [180, 181]. IL-7, however, appears also to have a detrimental effect on B cells, as high IL-7 levels were associated with an increased ratio

of immature transitional B cells in the blood of HIV-1 infected individuals during CD4+ T lymphocytopenia, as well as during IL-7 therapy [165, 182].

Although peripheral B cells do not express IL-7Rα, an indirect role for IL-7 in influencing the biology of mature B cells has been shown. IL-7 treated T cells produce IFN-γ which, in turn, induces the upregulation of CD95 on B cells; through this mechanism, the sensitivity of B cells to CD95 mediated apoptosis increases [183]. In **paper II,** we further describe the role of IL-7 in regulating B cell activation and survival through mechanisms which involves activation of T cells and production of B cell survival factors from T cells.

B cells co-cultured with T cells pre-treated with IL-7 for 5 days produced measurable levels of IgM and IgG, which were slightly higher than the levels found in cultures of B cells cultured in presence or absence of IL-7, or co-cultured with T cells without IL-7 (Figure 9A). This effect was more pronounced after 10 days of co-culture of B cells with IL-7 treated T cells; IL-7 treated T cells induced the secretion of IgM, IgG and IgA similar to the levels detected upon CD40L stimulation (**paper II**).

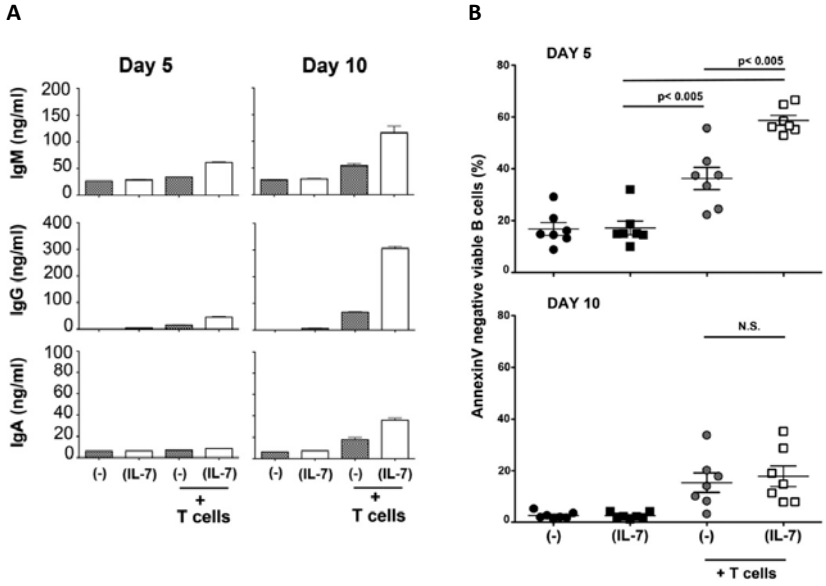

Figure 9. Immunoglobulin production and survival of peripheral B cells in the presence of IL-7 treated T cells. B cells were cultured for 5 or 10 days, alone or with IL-7, or co-cultured with T cells treated or not with IL-7. Immunoglobulin concentrations were measured in culture supernatants by ELISA (**A**) and B cell survival was analyzed using Annexin V staining (**B**).

B cells cultured alone, or in the presence of IL-7, usually die after 5 days. The presence of T cells in culture, however, increased the survival of B cells; in addition, B cells in co-culture with IL-7 treated T cells displayed further enhanced viability. This additional effect of IL-7 was lost at day 10, with equal effect on B cell survival in presence of IL-7 pre-treated or non-treated T cells (Figure 9B).

Next we studied the phenotype and proliferative capability of B cells co-cultured with IL-7 treated or non-treated T cells. In co-culture with IL-7 treated T cells, B-cell proliferation was detected. These proliferating B cells down-regulated CD20 and expressed higher CD38 levels, characteristic features of activated plasmablasts. CD20-CD38+ B cells were significantly increased in cultures with IL-7 pre-treated T cells. These results demonstrated that IL-7 treatment induces changes in T cells, which in turn enhance B cell activation and differentiation toward a plasmablast phenotype. Accordingly we searched for the molecular mechanism behind this finding.

There are different co-stimulatory pathways involved in B cell activation during an immune response. CD27, a member of the TNFR family, is a marker of memory B cells and an important receptor for T-dependent B cell responses [184]. CD27 triggering by its ligand, CD70, is involved in B cell differentiation toward plasma cell and Ig secretion. CD70 was upregulated on T cells in the presence of IL-7, as shown in Figure 10A.

BAFF is an important molecule for survival of B cells [74]. IL-7 induced BAFF production by T cells, as measured by ELISA (Figure 10B). In order to evaluate the role of BAFF on IL-7 induced B cell survival, we blocked BAFF using a soluble BAFFR in cultures of B cells and IL-7 pre-treated T cells. BAFF neutralization strongly decreased the effect of IL-7 treated T cells on B cell survival (Figure 10C), indicating that IL-7 promotes B cell survival via the induction of BAFF from T cells.

We compared the effects of CD70 and BAFF neutralization on antibody production using the cultures of B cells and IL-7 pre-treated T cells described above. CD70 neutralization induced a significant reduction of IgG production both in the presence and absence of BAFFR. On the contrary, the presence of BAFFR in the B-T co-cultures did not affect IgG production. In addition, the effect of anti-CD70 blocking antibody was not enhanced by the presence of BAFFR (Figure 10D). A comparable effect of CD70 blocking was seen for IgM production. Taken together, these results indicate that IL-7 regulates B cell activation and survival via two distinct mechanisms, by inducing CD70 up-regulation and BAFF production in T cells.

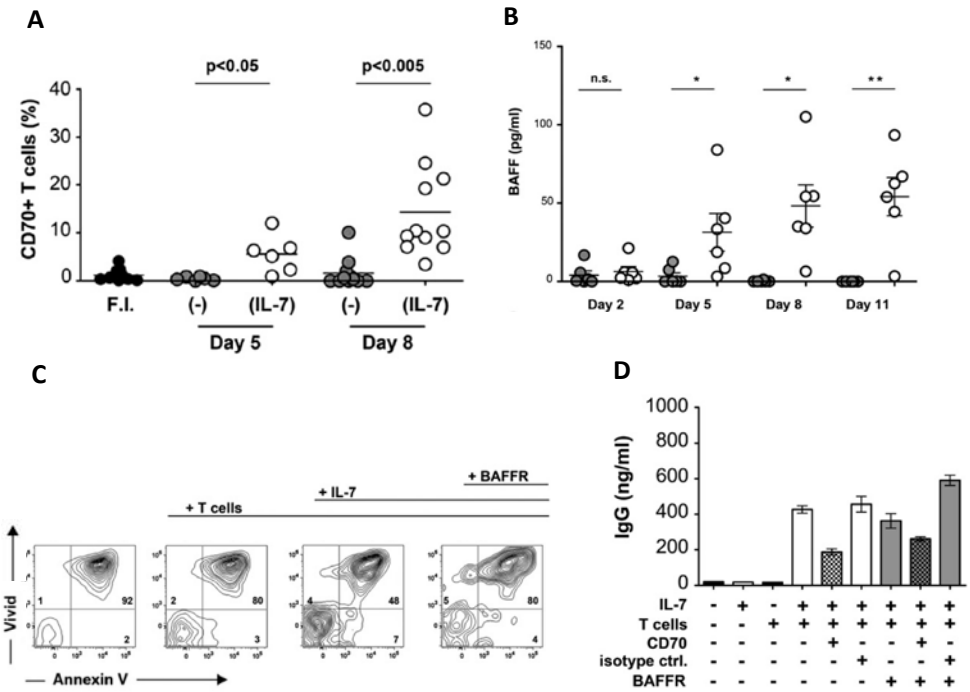

Figure 10. Contribution of CD70 expression and BAFF production from IL-7 treated T cells to B cell activation and survival. Percentage of CD70+ T cells among freshly isolated T cells (black dots) or T cells cultured in the presence (white dots) or absence (grey dots) of IL-7 for 5 or 8 days (**A**). BAFF concentration measured in the supernatant of T cells in the presence (white dots) or absence (grey dots) of IL-7 (**B**). Representative FACS plot is shown for B cells stained with Vivid and Annexin V after culture for 5 days alone, with untreated T cells, IL-7 treated T cells or with IL-7 treated T cells and recombinant BAFFR (**C**). B cells were cultured alone, in the presence of IL-7, untreated T cells or IL-7 treated T cells for 5 days and the levels of IgG antibodies were measured in the supernatants. The contribution of CD70 and BAFF in the activation of B cells was studied using CD70 neutralizing antibodies (CD70) and soluble BAFFR respectively in the presence of IL-7 treated T cells (**D**).

These results assign a functional role to CD70 expressed on T cells in inducing increased IgG production. Prior to ART it was clearly shown that T cells from HIV-1 infected patients have an increased CD70 expression [185]. Our group, and an additional paper published the same year, previously reported that CD70, ligand for CD27 molecule expressed on memory B cells, led to an increased IgG production and loss of memory B cells in HIV-1 infected patients [169, 186]. It is a well-known phenomenon that increased IgG level can be found in the serum of HIV-1 infected patients not receiving treatment. The mechanisms underlying B-cell polyclonal activation and hypergammaglobulinemia during HIV-1 infection remain poorly understood and the contribution of increased CD70 expression on T cells to this phenomenon promoted the following study, denominated as **paper III**.

5.3 PAPER III - Altered CD70 expression on T cells and its potential contribution to B cell abnormalities during HIV-1 infection

In paper II, we could show that CD70 molecules expressed by CD4+ T cells stimulate memory B-cell activation and antibody production in a non-antigen specific manner *in vitro*. In the context of HIV-1 infection, such mechanisms might theoretically be implicated in B cell activation and the increased IgG production, which is named as hypergammaglobulinemia, detected in patients. To clarify the involvement of these molecules in B cell activation during HIV-1 infection, CD70 expression on T cells and the functional and migratory properties of the CD4+CD70+ T cells were analyzed in paper III, in the following cohorts of HIV-1 infected individuals matched for age and gender and non-infected controls:

1) 20 HIV-1-infected patients naive to ART with CD4+ T-cell counts higher than 350 cells/µl (mean = 600 ± 271 cells/µl) and viral load ranging between 200 and 132 000 (median = 21 500) copies/ml. This group is defined as viremic.
2) 25 patients receiving ART and characterized by undetectable viraemia (<25 copies/ml) and CD4+ T-cell counts higher than 200 cells/µl (mean = 497 ± 222 cells/µl). This group is defined as non-lymphopenic, non-viremic.
3) 10 ART-treated CD4+ T-cell lymphopenic patients, with CD4+ T-cell counts below 200 cells/µl for a period of 3–20 years. Viral load ranged in these patients between undetectable and low (<100 copies/ml) levels, mean CD4+ T-cell count was 145 ± 65 cells/µl. This group is defined as lymphopenic.
4) 10 non-infected controls matched for age and gender to the HIV-1 infected cohorts.

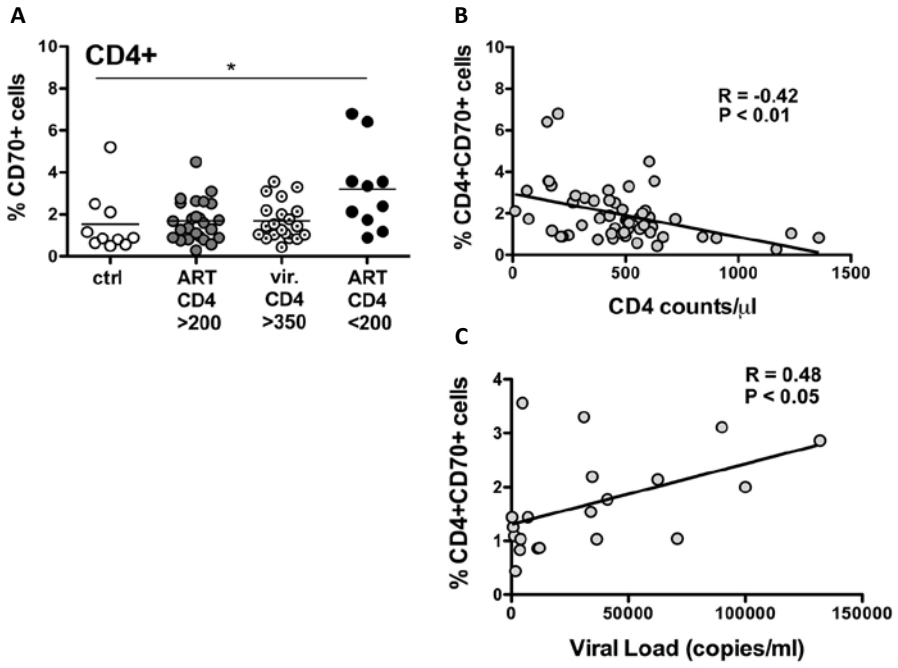

Figure 11. **CD70 expression on CD4+ T cells in HIV-1 infected and non-infected individuals in relation to CD4+ T cell counts and viremia.** CD70 expression was analyzed, using flow cytometry, on CD4+ T cells (**A**) of non-infected individuals (ctrl) and HIV-1 infected patients. The correlation is shown between CD4+ T cell counts in HIV-1 infected individuals and prevalence of CD70+ cells among all circulating CD4+ T cells (**B**). The frequency of CD4+CD70+ T cells correlates with VL in viremic HIV-1-infected patients (**C**).

We detected a significantly increased frequency of CD4+CD70+ T cells in lymphopenic patients as compared to non-infected individuals (Figure 11A). An inverse correlation was observed between CD4+ T cell counts and the frequency of CD4+CD70+ T cells in the whole group of HIV-1 infected patients (Figure 11B); in addition, the levels of CD4+CD70+ T cells correlated with VL within the group of viremic individuals (Figure 11C), indicating a potential contribution of high viremia in modulating CD70 expression.

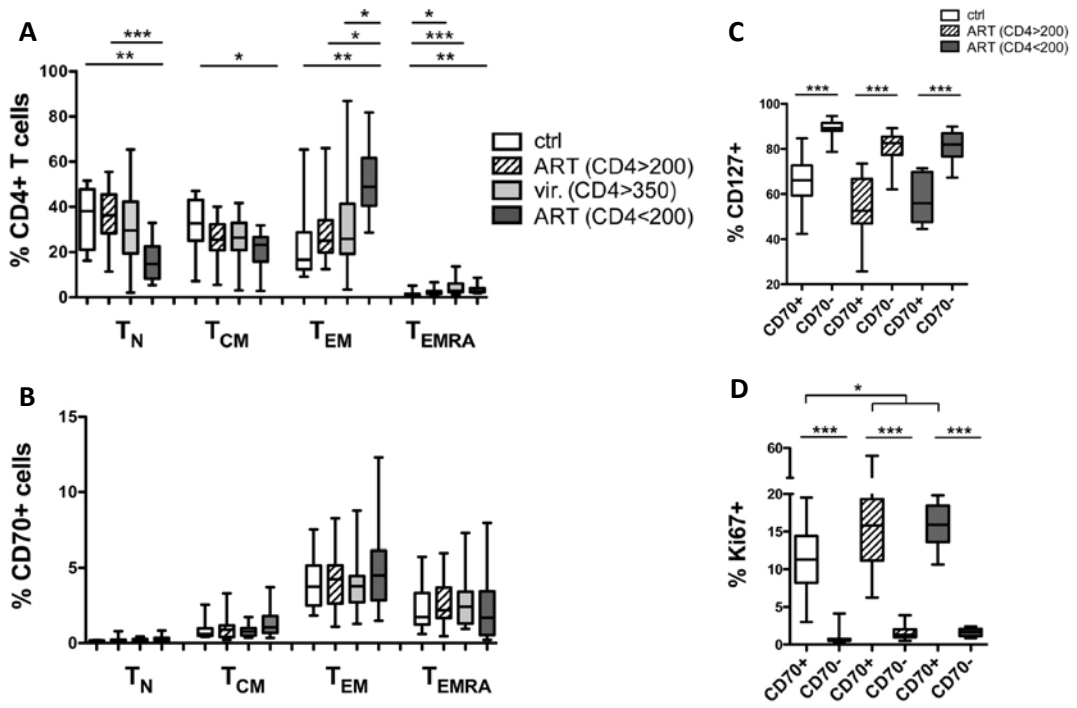

Figure 12. T cell activation and proliferation regulates the size of the CD4+CD70+ T cell populations. The ratio of the different naïve and memory subsets among CD4+ T cells of HIV-1 infected and non-infected controls (ctrl) is shown (**A**). CD70 expression was measured on CD45RA+CCR7+ naïve, CD45RA-CCR7+ central memory (CM), CD45RA-CCR7- effector memory (EM) and CD45RA+CCR7- effector memory (TEMRA) CD4+ T cells of HIV-1 infected and non-infected controls (**B**). The expression of CD127 (**C**) and the proliferation marker Ki67 (**D**) was compared between CD4+ CD70+ and CD4+ CD70- T cells from ART-treated patients and from non-infected controls.

Lymphopenic patients were characterized by a significantly increased frequency of CD4+ T_{EM} subset as compared to non-infected controls and the additional groups of HIV-1 infected patients (Figure 12A), suggesting that the increased frequency of CD4+CD70+ T cells in lymphopenic patients might be due to an accelerated naïve-to memory differentiation. CD70 was preferentially expressed on CD4+ T_{EM} cells, and to a lesser extent, on CD4+ $T_{EMRA,}$ both in HIV-1 infected patients and control subjects (Figure 12B); these results indicated that a higher CD70 expression in lymphopenic patients may be associated with the increased frequency of effector memory CD4+ T cells. CD4+ CD70+ T cells expressed reduced levels of CD127 (Figure 12C) and increased levels of the proliferation marker Ki67 (Figure 12D) as compared with the CD4+ CD70- counterpart, suggesting that CD4+CD70+ T cell frequencies might increase in lymphopenic and viremic patients due to accelerated T cell activation.

In the course of HIV-1 infection, CD4+ T cell depletion is often associated with increased IL-7 levels. In the cohort of lymphopenic patients included in the present study we previously detected low levels of IL-7 [174]; this result might be explained by the prolonged lymphopenia and its harmful effects on lymphoid niches leading to a reduced availability of IL-7 which results in increased apoptosis and loss of mainly naive T cells [187]. Importantly, we did not detect any correlation between plasma IL-7 levels and CD70 expression; accordingly, CD70 upregulation in lymphopenic patients appears to occur independently of circulating IL-7 levels. It cannot however be excluded that IL-7 produced locally in lymphoid tissues may be involved in the CD70 up-regulation which was noticed in effector memory cells. It is of interest that when studying different factors which could be involved in CD70 up-regulation on T cells we found that IL-2, IL-7 and TCR triggering led to CD70 up-regulation in T-cells from HIV-1 infected patients and controls.

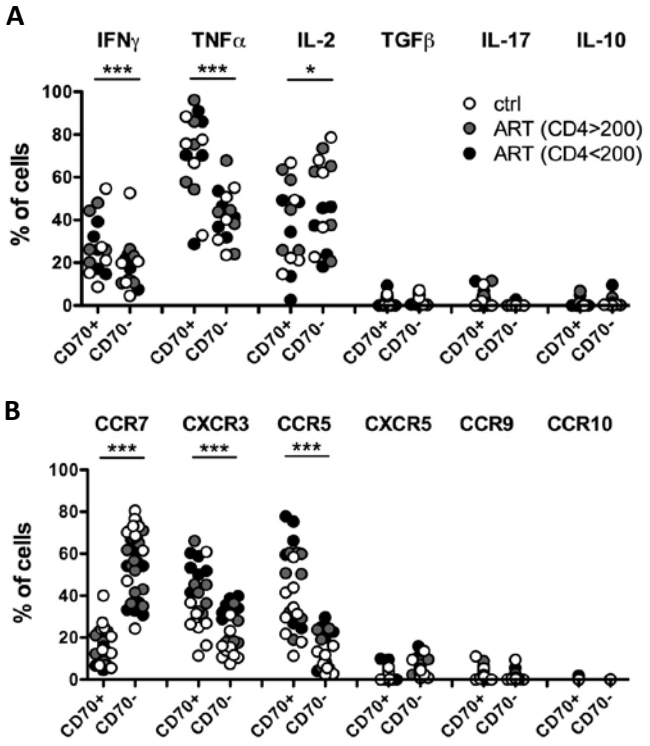

Figure 13. **CD4+CD70+ T cells produce Th1-type cytokines and express chemokine receptors mobilizing them towards sites of inflammation**. The production of the cytokines IFNγ, TNFα, IL-2, TGFβ, IL-17 and IL-10 from CD4+ CD70+ and CD4+ CD70- T cells of treated HIV-1 infected patients and in non-infected controls was studied, following stimulation with PMA and Ionomycin (**A**). We also evaluated the expression of CCR7, CXCR3, CCR5, CXCR5, CCR9 and CCR10 chemokine receptors on these cells (**B**).

CD4+CD70+ T cells of HIV-1 infected patients, as compared with the CD4+ CD70- population, produced at a higher frequency the pro-inflammatory cytokines IFN-γ, TNF-α and at a lower frequency IL-2 (Figure 13A). The migratory profile of the CD4+ CD70+ T cells included a higher expression of CCR5 and CXCR3 as compared with their CD70- counterparts, suggesting their homing property to inflamed tissues in both HIV-1 infected and non-infected individuals. The low CCR7 and CXCR5 expression indicated that the CD4+CD70+ T cells may act outside the lymphoid follicles and the lack of CCR9 and CCR10 expression indicated no selected homing properties of the CD4+CD70+ T cells to the intestine or the skin, respectively (Figure 13B).

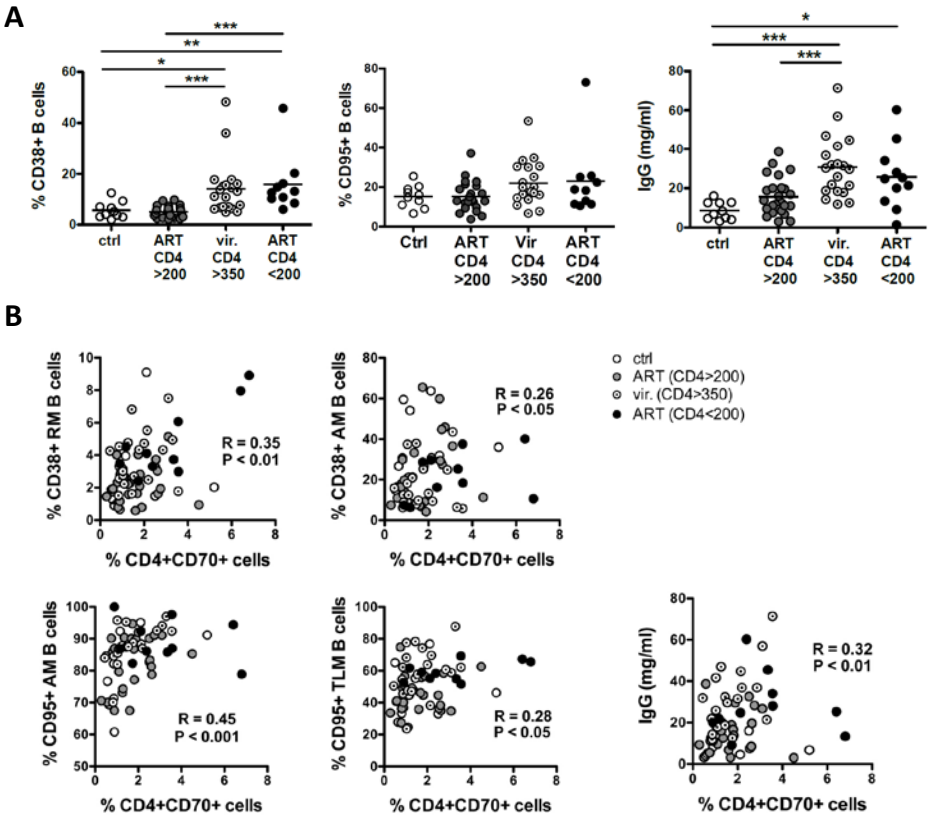

Figure 14. **Marker of B cell activation, apoptosis and plasma IgG levels correlate with CD4+CD70+ T cell frequencies.** The frequency of CD38 and CD95 expression on B cells and the levels of IgG in plasma were measured in specimens from non-infected controls (ctrl) and HIV-1 infected patients (**A**). We analyzed the association between CD4+CD70+ T cell frequencies and the expression of CD38, CD95 and plasma IgG levels (**B**) on resting memory (RM), activated memory (AM) and tissue-like memory (TLM) B cell subsets in the same groups of individuals.

We analyzed whether the increase of CD4+ CD70+ T cell frequencies in circulation can be associated with phenotypic alterations of B cells. A higher expression of the activation marker CD38 on B cells was detected in both viremic and lymphopenic patients, as compared with non-infected controls or non-lymphopenic, non-viremic patients (Figure 14A). When all subjects were analyzed together, the percentage of CD4+CD70+ T cells correlated with CD38 expression on RM B cells and AM B cells (Figure 14B). In addition, CD95 expression among AM and TLM B cells correlated with the frequency of CD4+ CD70+ T cells (Figure 14B). These results indicated that CD70 expression on T cells may possibly be a driving force for activation of memory B cell subsets, as measured by CD38 and CD95 expression.

The IgG concentration in plasma was elevated in viremic and lymphopenic patients compared with non-infected controls (Figure 14A), whereas the levels of plasma IgG in serum of non-lymphopenic, non-viremic patients were similar to what found in the controls. The frequency of circulating CD4+CD70+ T cells correlated with plasma IgG levels when all groups of patients and controls were analyzed together (Figure 14B). Since we found that CD38 expression and plasma IgG levels were all upregulated in viremic patients, similarly to what observed in lymphopenic patients, it is likely that viremia-induced factors can contribute to the correlation noticed between CD4+CD70+ T cells and parameters of B cell activation. However once we excluded the viremic patients from our analysis we still found that CD4+CD70+ T cell frequency significantly correlated with CD38 expression on RM and AM B cells, CD95 expression on AM and TLM B cells as well as IgG levels in plasma (supplementary Figure 4 in paper III). B cell proliferation was studied in HIV-1 infected individuals and non-infected controls and we detected a correlation between Ki67 expression on B cells and CD4+ CD70+ T cell frequency.

In conclusion we found that CD70+CD4+ T cells correlate to CD4+ T cells counts in HIV-1 infected individuals. While we could not firmly pin-point the mechanism for this CD70 upregulation it is possible that the inflammatory environment caused by virus replication in several lymphoid organs may be at the basis for the upregulation of CD70 molecule on T cells. CD70+CD4+ T cells were mostly found among highly differentiated T_{EM} and T_{EMRA} cells. The expression of CD70 on CD4+ T cells correlated to activation markers present on populations of memory B cells and serum IgG levels. It is of interest that in patients with SLE, a disease characterized by abnormal activation of B cells and autoantibody production, an increased frequency of CD70+CD4+ T cells is found [188]. HIV-1 infection leads to increased production of circulating IgG; although a detailed characterization of the specificity of these antibodies is still missing it is believed that a large portion of circulating IgG during HIV-1 infection may

represent auto-antibodies with unknown targets. These findings create an interesting parallel between the auto-immune disease SLE and the chronic HIV-1 infection.

If CD70+CD4+T cells have a role in B cell activation, as suggested by the ex-vivo analyses shown in **paper III**, it is unclear at which site CD70+ CD4+T cells promote B cell activation. As CD70+CD4+T cells appears to be equipped with chemokine receptors ensuring their migration to inflamed tissue, one possibility is that CD70+CD4+T cells promote B-cell activation in inflamed tissues.

5.4 Paper IV - ART initiated during primary HIV-1 infection and its impact on the establishment of abnormal phenotypic features of CD4+ and CD8+ T cells

Although ART can achieve a complete or nearly complete suppression of HIV-1 replication, prevent AIDS and reduce overall mortality, antiviral drugs do not fully target immune activation and residual immune activation persists even after many years of viral suppression [129, 133, 189]. Chronic immune activation is considered to be the driving force of HIV-1 pathogenesis and is giving rise to phenotypic dysfunctions in HIV-1 specific and non-HIV-1 specific CD4+ and CD8+ T cells [133]. The phenotypic dysfunctions include distinct or combined features of abnormal immune activation, senescence and inhibition of immune responses. In this cross-sectional study, we included two groups of HIV-1 infected patients who had previously begun ART during PHI or in the chronic phase of infection. We aimed at investigating whether early ART initiation prevents the establishments of abnormal phenotypic features reported in CD4+ and CD8+ T cells of patients treated in the chronic phase of infection.

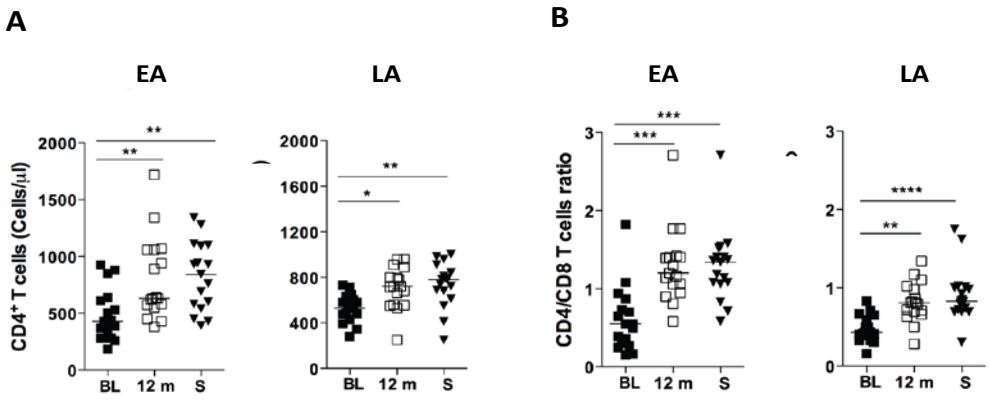

Figure 15. CD4+ T cell counts (A) and CD4/CD8 ratio (B) in individuals receiving ART during early and late phases of HIV-1 infection. EA=early ART; LA=late ART. The measurements were performed before ART initiation at baseline (BL), at 12 months (12m) from ART initiation and at sampling (S).

The early treated group, EA and the late treated group, LA, both consisted of 17 patients (16 males: 1 female); the mean age and SD was 43.8± 14.9 for the EA group and 42 ± 11.2 for the EA group. At the time of specimen collection, both patient groups had received treatment for a comparable period of time; individuals in the EA group had been treated for 25 months (range 7-59) and individuals in the LA group for a median of 29 months (12-60). In the cohort of EA patients, 11 were classified as Fiebig stage II, 3 as stage IV and 3 additional patients as stage V. The control group, named C, consisted of 25 non-infected subjects (all men); in this control group the mean age and SD was 40.4 ±14.0.

At baseline, CD4+ T cell counts were lower in the EA group, as compared with the LA group; at seroconversion, the time-point when viremia peaks, there is often a massive depletion of CD4+ T cells. In addition, at baseline, the virus titers in the EA group were higher than in the LA group, with a HIV-1 RNA value (copies/ml) of 5.97 log in the EA group as compared with 4.57 log in the LA group. At 12 months from the initiation of ART and at time of sampling, the virus was undetectable (<20 copies/ml) in the blood of all HIV-1 infected individuals. As assessed by the clinical records, the absolute numbers of CD4+ T cells increased in both the EA and LA groups at 12 months after initiation of ART. At the time of sampling, CD4+ T cell count had further increased in both groups of patients (Figure 15A).

The median CD4/CD8 T cell ratio increased over time in both the EA and LA groups; however the increase was more significant in the EA group, and the median CD4/CD8 T cell ratio in the LA group never reached the value of 1 or above at the time point for sampling (Figure 15B). Low numbers of naïve and circulating CD4+ T cells, high numbers of differentiated memory CD8+ T cells and chronic inflammation are associated with immunosenescence in elderly people, features also common in HIV-1 infection [89, 190]. A low CD4/CD8 ratio is associated with T cell activation, senescence, and chronic inflammation [191, 192] and has also been linked to a poor prognosis in ART-treated HIV-1 infected individuals [193]. A role for CD4/CD8 T cell ratio as a morbidity biomarker has been proposed as a recent study showed that, in spite of CD4+ T cell count normalization in the majority of patients on long-term ART, an increased risk of mortality still persists for this group of patients; this risk might be predicted by the persistence of a low CD4/CD8 T cell ratio in spite of increased CD4+ T cell counts [194]. Another study showed that the CD4/CD8 T cell ratio correlated with increased frequencies of CD4+ T cells expressing the activation markers CD38 and HLA-DR and the inhibitory receptor PD-1 [195], highlighting the CD4/CD8 T cell ratio as a surrogate marker for CD4+ T cell dysfunction in HIV-1 infection.

We studied the frequency of total CD4+ and CD8+ T cells and their subpopulations in blood. The frequencies of CD4+ T cells in the LA group were significantly lower as compared with the control subjects. The frequency of total CD8+ T cells was higher in both patient groups as compared with controls. The frequencies of TEMRA CD4+ T cells were statistically increased in the EA and LA groups as compared with controls. Among CD8+ T cells, the frequencies of TEMRA+ cells in the LA group were significantly higher as compared with the control group. Of note, no difference was detected for the frequencies of the studied CD4+ and CD8+ T cell populations between the EA and LA groups.

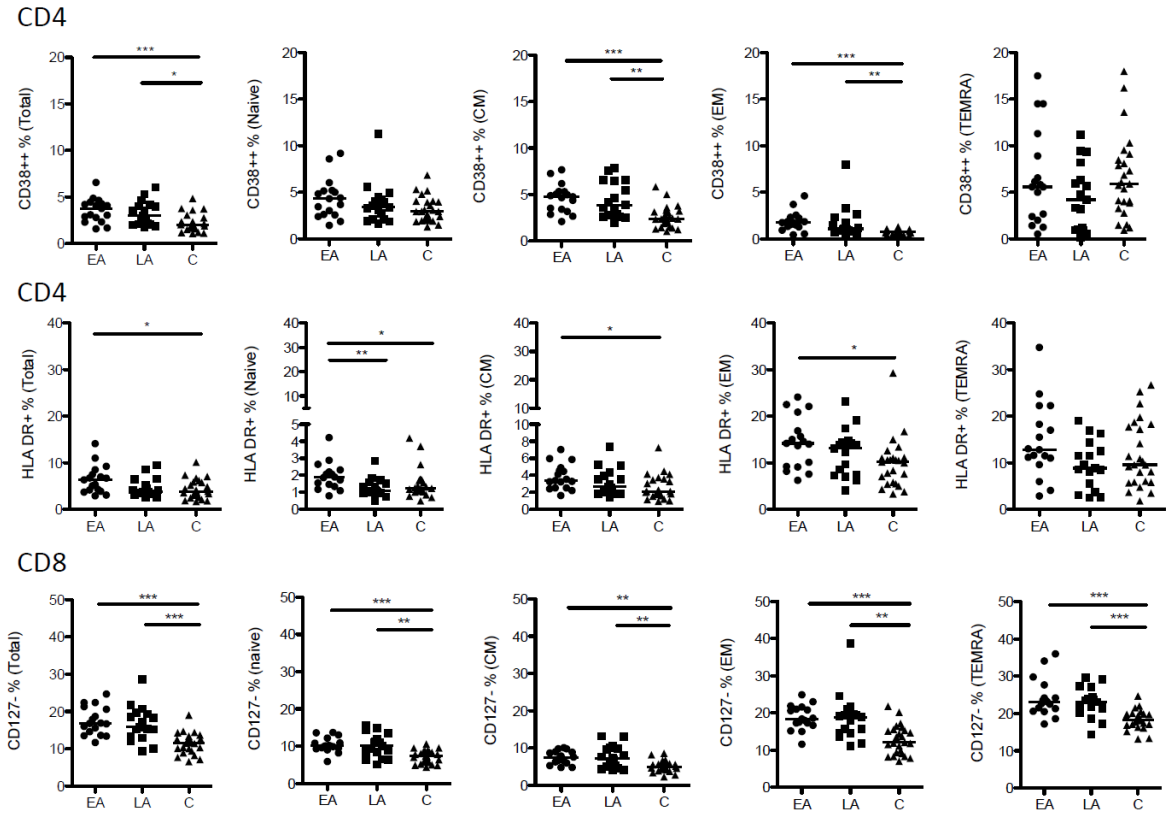

Figure 16. Frequency of CD38++ CD4+, HLA-DR+ CD4+ and CD127- CD8+ T cell subsets. The frequency of CD38++ and HLA-DR+ cells among total, naïve, central memory (CM), effector memory (EM) and effector memory CD45RA+(TEMRA) CD4+ T cells is shown, together with the frequency of CD127- total, naïve, CM, EM and TEMRA CD8+ T cells.

The expression of different CD4+ and CD8+ T cell markers associated with HIV-1 pathology was studied. These included immune activation (HLA-DR, CD38), senescence (CD28, CD57) exhaustion (PD-1) and terminal differentiation (CD127). A higher frequency of total CD4+ T cells, naïve, CM and EM CD4+ T cells from the EA group were HLA-DR+ as compared with the corresponding cells and subpopulations in the control group. When comparing the two groups of HIV-1 infected individuals, a higher frequency of HLA-DR+ naïve CD4+ T cells was found in the circulation in the EA group as compared with the LA group. In addition, higher levels of total CD4+ T cells, CM and EM CD4+ T cells from both patient groups were CD38++ as compared with controls. A higher frequency of CD127- cells was found in total CD8+ T cells and in all subpopulations of CD8+ T cells of EA and LA as compared with the control group (Figure 16). Markers used for identifying senescent cell populations were both upregulated in

different CD4+ T cell populations in the LA patient group; a larger frequency of CD28- total, EM and TEMRA CD4+ T cells were identified in the LA group as compared with the control group. In addition, CD57 was also expressed at higher levels on EM and TEMRA CD4+ T cells from the LA group versus control group.

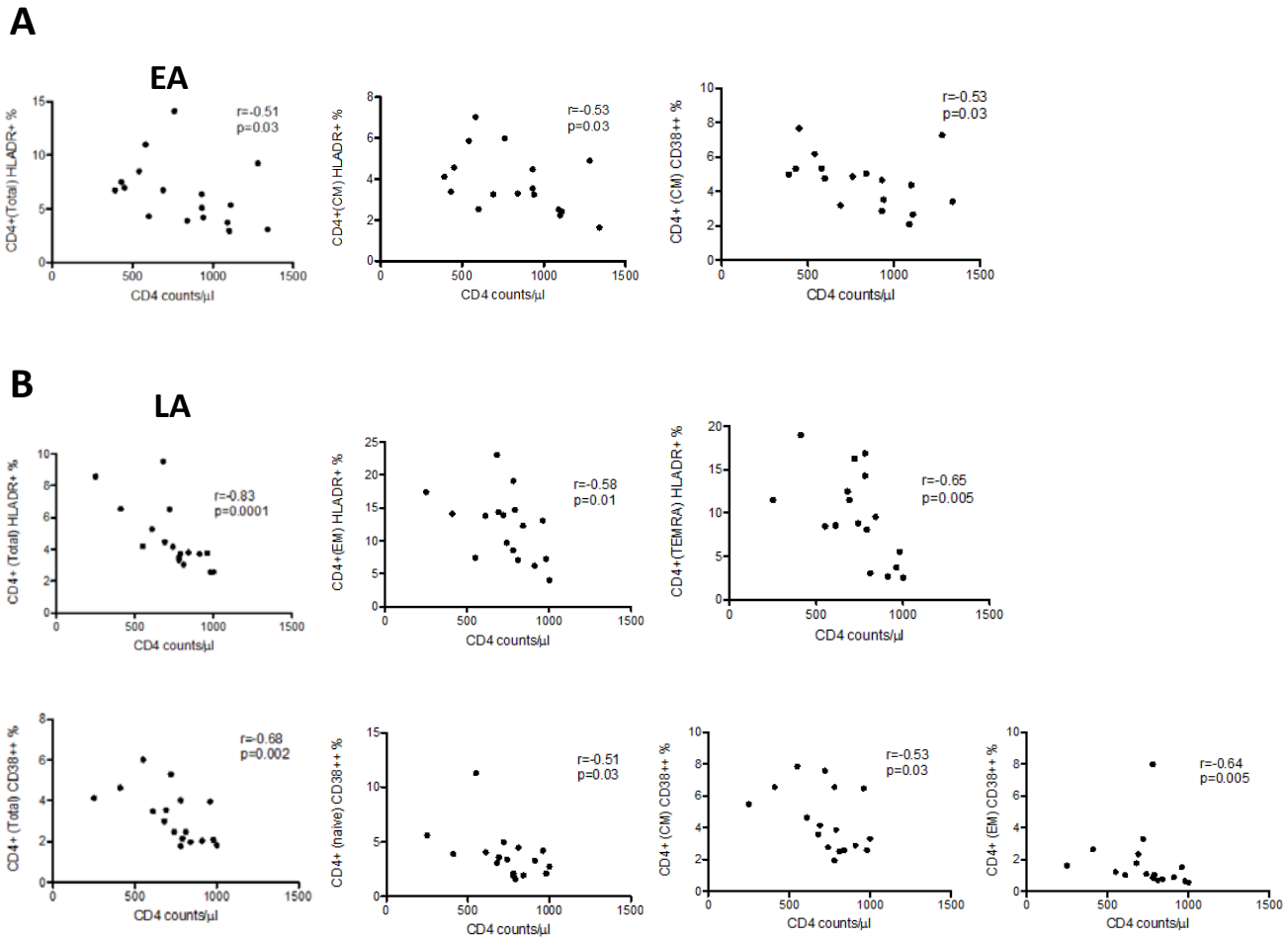

Figure 17. Activated CD4+T cells in HIV-1 infected patients are associated with CD4 +T cell counts. In specimens from LA patients, the frequency of HLA-DR+ total, EM and TEMRA CD4+T cells, and CD38++ total, naïve, CM and EM CD4+ T cells inversely correlated with lower CD4 T cell counts/µl (**A**). In specimens from EA patients, the frequency of HLA-DR+ in total and CM CD4+ T cells and CD38++ CM CD4+ T cells inversely correlated with CD4+ T cell counts/µl (**B**).

Ki67 is an intracellular marker widely used to characterize proliferating cells. A larger frequency of CM and EM CD4+ T cells from both HIV-1 patient groups expressed Ki67, as compared with the control group. Furthermore, an indirect correlation was found between the CD4+ T cell

counts and the frequency of Ki67+ total CD4+ T cells, Ki67+ CM CD4+ T cells and Ki67+ EM CD4+ T cells from LA patients. Similarly, CD4+ T cell counts from the same LA group inversely correlated with frequency of Ki67+ total CD8+ T cells, Ki67+ CM CD8+ T cells and Ki67+ EM CD8+ T cells. These results suggest that proliferating T cells may have a negative effect on the absolute CD4+ T cell number, even in treated patients.

We measured the levels of soluble markers of immune activation and inflammation in plasma. Significant levels of inflammatory markers distinguishing the groups of patients from one another could not be detected, which is likely due to the positive effect of ART in both groups of patients. However, the levels of sCD14 were significantly higher in the group of EA patients as compared with the control group; the median levels of sCD14 were also higher in the LA group compared to controls although did not reach a significant difference. Furthermore, the levels of β2M were higher in the LA group as compared with the control group. β2M has been shown to be increased during HIV-1 infection and released by activated T cells [146], data consistent with our findings on persistent T cell activation.

In the LA group, the expression of HLA-DR+ and CD38++ in most CD4+ T cell subpopulations negatively correlated with the CD4+ T cell counts (Figure 17A). In the EA group, HLA-DR+ total and CM CD4+ T cells and CD38++ CM CD4+ T cells inversely correlated with the CD4+ T cell counts (Figure 17B). The lack of CD127 expression on CD8+ T cells and CD8+ T cell subpopulations from the EA group inversely correlated with the CD4/CD8 T cell ratio, whereas lack of CD127 expression on CD8+ T-cell subpopulations from the LA group did not correlate with this parameter. It has been shown that CD4+ T cell counts often fail to return to normal levels and inflammation and T- cell activation remain elevated during ART administration [103, 189]. Also in our study, the expression of the activation markers HLA-DR and CD38 were upregulated on CD4+ T cells and inversely correlated with the CD4 + T cell counts, supporting the observation that the high activation levels of T cells are independent predictors of CD4+ T cell decline and progression to AIDS and therefore a continuous driving force fueling HIV-1 pathogenesis [196].

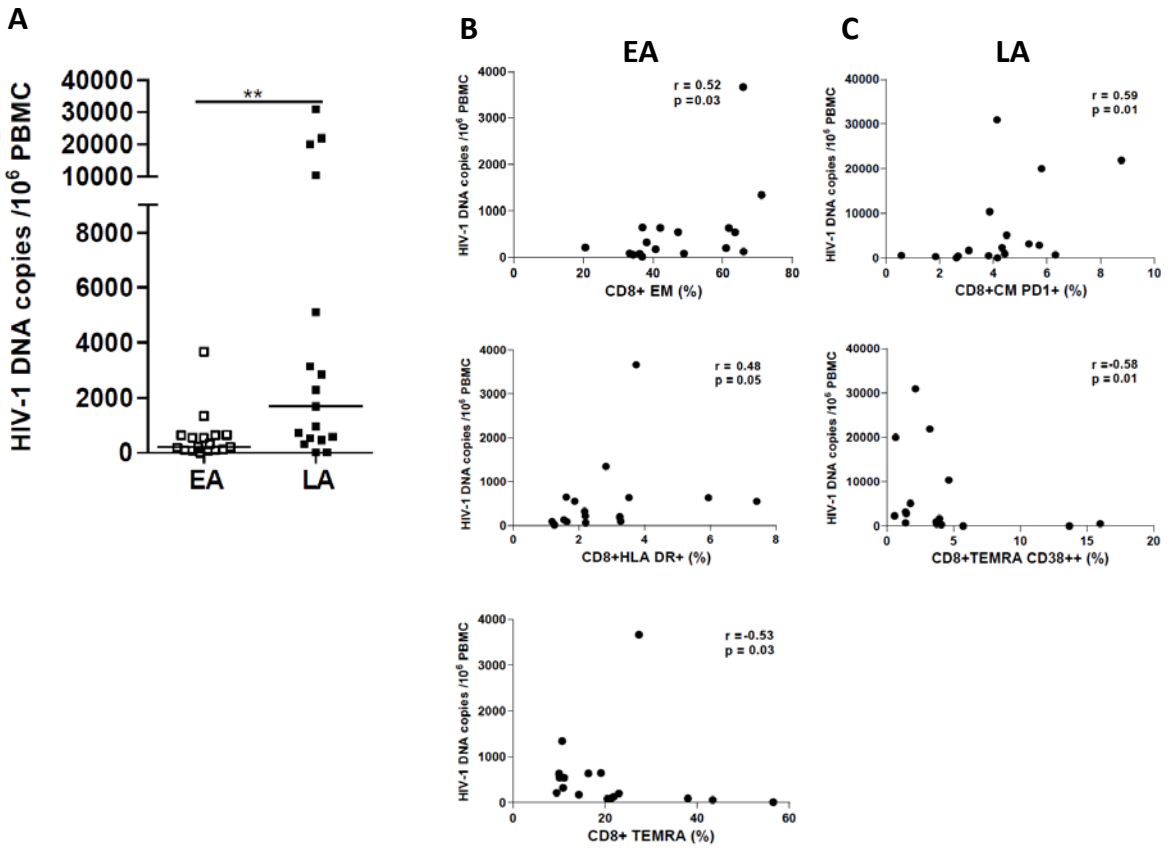

Figure 18. Size of total HIV-1 DNA copies and its correlation to T cell subpopulations and surface markers. Copies of HIV-1 DNA in PBMCs from EA and LA patients (**A**). In specimens from EA patients, the number of HIV-1 DNA copies was shown to directly correlate with the frequencies of CD8+ EM and CD8+ total HLA-DR+ T cells, whereas a negative correlation was shown between the size of virus reservoir and the frequency of CD8+ TEMRA T cells (**B**). In specimens from LA patients, the number of HIV-1 DNA copies, directly correlated to the frequency of CD8+ CM PD-1+ T cells and indirectly to the CD8+ TEMRA CD38++ T cells (**C**).

We determined the size of the virus reservoir in EA and LA patients by the quantification of total HIV-1 DNA in PBMCs. The copies of HIV-1 DNA detected in PBMCs of EA patients treated during the acute phase of infection were significantly lower than what was found in LA patients treated during the chronic phase of infection (Figure 18A). This result confirms a previous study on the beneficial effect of early ART on confining the establishment of virus reservoirs [197]. A positive correlation was found between the total HIV-1 DNA copies of EA patients and the frequencies of CD8+ EM and CD8+HLA-DR+ T cells, whereas an indirect correlation was detected with CD8+ TEMRA+ T cells (Figure 18B). In the LA group, the copies

of HIV-1 DNA correlated with the frequencies of CD8+ CM PD-1+ T cells and inversely with CD8+ TEMRA CD38++ T cells (Figure 18C). It is not surprising that PD-1 and HLA-DR, markers of exhaustion and immune activation respectively, directly correlated with the size of HIV-1 DNA as they might be involved in sustaining virus replication by suppressing immune functions.

We have shown that features of immune activation, exhaustion and terminal differentiation are present on CD4+ and CD8+ in patients treated during PHI. Their dysfunctional phenotype does not distinguish them from patients who started ART during the chronic phase of infection. Importantly, these abnormalities are similar in the two patient groups despite the significant difference in the number of total HIV-1 DNA copies in PBMCs, with lower amounts in EA patients. In addition, it cannot be excluded that despite successful ART, the size of the virus reservoir may be different in relevant lymphoid tissues where the abnormal immune activation takes place. It is highly relevant to identify biomarkers which may predict immunological preservation in patients treated during PHI; these patients may be part of cohorts selected to assess new therapy to cure HIV-1 infection.

5.5 Preliminary results - Frequency of B cell sub-populations in the blood of EA and LA HIV-1 infected patients

Many of the B cell defects described to occur in HIV-1 viremic individuals can be improved with ART, although the normalization of the frequency of some B cell subsets has been shown to be slow and incomplete in patients initiating ART during the chronic phase of infection [152, 176, 177]. We aimed at investigating the frequency of B cell subsets in our cohort of early and late treated HIV-1 patients included in **paper IV**. Our group and others have previously shown that ART initiation in the early phase of HIV-1 infection is associated with improved B cell memory function as measured by memory B cell responses to different recall antigens in both adults and children [167, 170].

As compared to non-infected individuals, LA patients exhibited a significant decrease of total B cell frequency as compared to controls, a finding which was not present in the group of EA patients (Figure 19). We could not find any significant difference between the groups of HIV-1 infected subjects and controls in relation to the frequencies of IT, Naïve, RM, AM, TLM and MZ-like (CD19+CD10-CD27+IgM+IgD+) B cells. There was however a tendency for AM B

cells to be higher in the groups of HIV-1 infected subjects (6.64±4.33, 6.82±3.83 and 4.4±2.01 in EA, LA and control subjects respectively) as compared to controls, (Figure 19A).

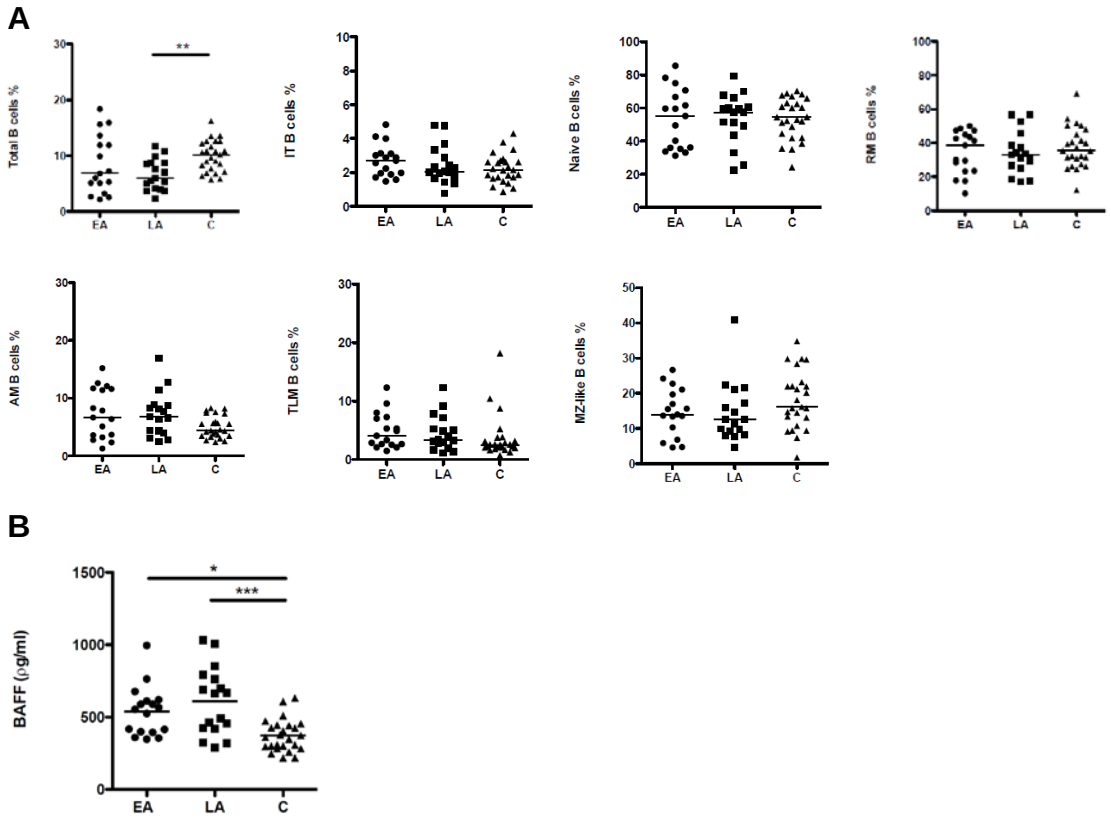

Figure 19. B cell phenotype of early and late treated HIV-1 infected patients. The phenotype of B cells was assessed on PBMCs from EA and LA HIV-1 infected patients and in non-infected subjects. B cell subpopulations were gated on living CD19+ B cells as detected by the Live/Dead kit labeled with a near-infrared dye. Frequency of total B cells (CD19+), immature transitional (IT) B cells, naïve B cells, resting memory (RM) B cells, activated memory (AM) B cells, tissue-like memory (TLM) B cells and MZ-like B cells (**A**) as previously reported [155]. Levels of BAFF were measured in plasma samples by ELISA (**B**). Statistical analyses were performed using Kruskal-Wallis test followed by Dunn's post test.

It was surprising that the frequency of B cell subpopulations was not significantly different in the LA group as compared to controls; however this group of patients initiated treatment at a median CD4+ T cell count of 530 cells/µl which is relatively high compared to patients included in previous studies; the EA patients began ART at a median CD4+ T cell count of 430 cells/µl. That the frequency of B cell sub-populations was not different between patients in the EA group and control was less surprising, as the hope is to prevent immunological impairment by administrating ART during PHI. One limitation of our study is that the number of included

patients is low; the significance of our results may become more clearly by increasing the number of EA and LA patients. In addition we did not follow the patients longitudinally from the time of ART initiation.

Among the soluble factors analyzed in serum of the two groups of EA and LA patients and controls, we also measured the levels of BAFF. It was interesting that the levels of BAFF in our cohorts of EA and LA HIV-1 infected patients were significantly increased compared to controls; this difference was more pronounced between the LA group and controls (Figure 19B). It has previously been shown that BAFF is increased in the serum of HIV-1 infected patients; the increased level of BAFF was in some cases associated with viremia [198, 199] and also persisted in patients despite successful ART [200].

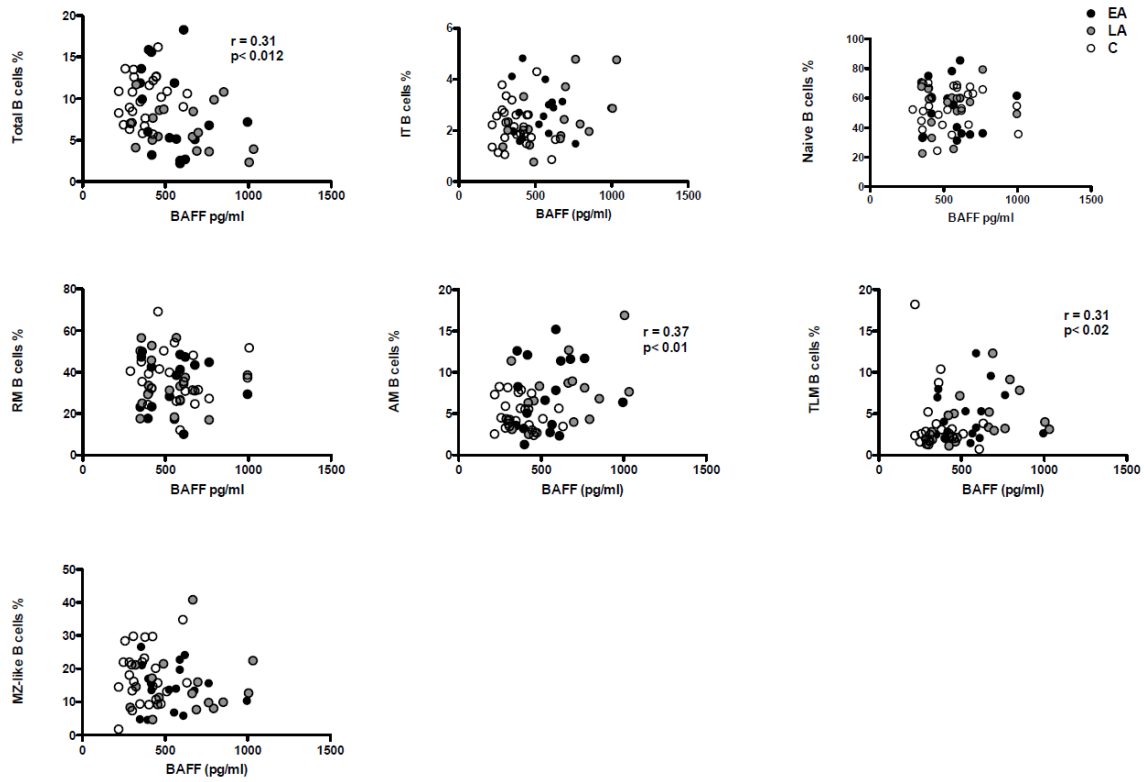

Figure 20. Frequencies of total and memory B cell sub-populations and their correlation with BAFF levels. We analyzed the potential correlation between the frequencies of circulating B cell sub-populations with the levels of BAFF in plasma from both EA and LA HIV-1 infected patient groups and controls. Data were analyzed using Spearman correlation test.

We related the frequency of B cell sub-populations in the whole group of EA and LA patients and controls to BAFF levels and the results of these correlations are shown in Figure 20. A positive correlation was found between the levels of BAFF and total B cells, AM B cells and TLM B cells. The role of BAFF in B cells dysfunctions during HIV-1 infection has not been thoroughly studied. A study conducted in SIV-infected RM, which exhibited elevated levels of BAFF, showed a consistent B cell polyclonal activation detected by IgM and IgG hyperglobulinemia and a significant increase in the relative frequencies of AM B cells and exhausted TLM B cells [215]. That elevated BAFF levels can be found in successfully ART-treated individuals suggest that early ART does not correct for this abnormality which may be consequence of persistent HIV-1 infection and chronic immune activation.

BAFF has been described as an important molecule in the context of SLE, as blocking of this molecule through a monoclonal antibody targeting BAFF ameliorates disease activity [79, 201]. Even in the context of SLE is not completely clarified the role of BAFF in mediating a disease characterized by alterations in B cell tolerance, caused by defective tolerance check-points. Excess of BAFF rescues autoreactive anergized cells and promotes their maturation into follicular or MZ B cells [201].

The mechanism leading to increased circulating levels of BAFF during HIV-1 infection is unknown. A recent study described a role for the viral factor Nef in promoting the high expression of BAFF by dendritic cells in the blood of HIV-1-infected individuals receiving ART, suggesting a role for persistent HIV-1 infection in driving the upregulated levels of BAFF in circulation [202]. It is interesting that in our cohort of EA patients, where the size of virus reservoir is limited and virus replication cannot be detected in blood, high levels of BAFF were anyway detected in circulation. A recent study highlighted the importance of macrophages as a source of BAFF since they are among the first cell populations targeted by the virus, following PHI. Once macrophages are infected they display an altered production of cytokines/chemokines which contributes to the general state of immune activation during HIV-1 infection, likely to affect both T and B cells [203].

6 CONCLUSIONS AND FUTURE DIRECTIONS

Within the first weeks of HIV-1 infection, the massive CD4+ T cell depletion taking place in the GALT is contributing to the impaired regulation of the epithelium and breakdown of the mucosal barrier. The severity of CD4+ T cell depletion is associated with microbial translocation, chronic immune activation and disease progression in HIV-1 infected individuals [104, 105]. Initiation of ART leads to suppression of HIV-1 replication in a large majority of patients, thus improving the immune function and nearly eliminating the risk of AIDS-related complications. Effectively treated HIV-1 infected individuals are, however, at higher risk of non-AIDS related morbidity than age-matched HIV-1 non-infected adults [31, 204].

Specimens from the patients included in my studies were collected starting in 2009; at that time point our clinical collaborators in Stockholm still followed several patients who did not receive ART as their CD4+ T cell counts was above 350 cells/μl. The situation is different today as in Sweden, following recent recommendation from WHO, HIV-1 infected patients will receive ART independently of their CD4+ T cell counts as soon as their HIV-1 status will become known. This is obviously a fantastic opportunity for the clinical management of HIV-1 infection as ART will confine inflammation and immune activation, thus ultimately leading to a healthier life for HIV-1 infected patients. This poses however some problems to conduct studies addressing the natural history of HIV-1 pathogenesis. Accordingly when discussing future perspectives within the frame of the studies which I have conducted, it has to be taken in account that specimens can only be obtained from ART treated patients.

In **paper I,** we found an increased expression of IL-21R on classical memory B cells (CD19+CD10-CD27+) and TLM cells from viremic HIV-1 infected patients. The expression of the IL-21R on RM B cells was shown to correlate with their decreased frequency in the circulation of HIV-1 infected patients, suggesting that IL-21R expression on B cells may play a role in compromising the survival of these cells. In line with this possibility, IL-21R positive B cells were found to be more susceptible to apoptosis, as shown by lower Bcl-2 expression. We unraveled two mechanisms which could lead to up-regulated expression of IL-21R during HIV-1 infection. Several TLR agonists directly triggered the expression of IL-21R on B cells and, in addition, the elevated levels of sCD14 in circulation correlated with IL-21R expression on RM B cells and the decreased levels of these cells in circulation. We also described an increased activation of B cells from HIV-1 infected individuals, as measured by CD38 expression on total and classical memory B cells, which was associated with HIV-1 replication. CD4+ T cell counts

were found to be inversely correlated with CD38 expression on B cells; this correlation was stronger in viremic patients and HIV-1 viral load was also shown to directly correlate with CD38 expression on B cells in HIV-1 patients. These findings suggest that microbial translocation and associated immune activation may contribute to loss of memory B cells during HIV-1 infection.

It is unclear whether the increased frequency IL-21R positive B cells in HIV-1 infected patients corresponds to an increased signaling through this receptor; it is highly possible that increased IL-21R expression only reflects activation of B cells by microbial components as shown in paper I. We measured the levels of IL-21 in serum and found that this cytokine was present at a reduced level in the plasma of HIV-1 infected subjects, independently of ART, as compared to controls. During the time our study was conducted another group reported the same finding on reduced level of IL-21 in HIV-1 infection [205]. Tfh cells are important producers of IL-21 and a reduced level of circulating IL-21 could reflect a reduced capacity of Tfh cells to produce IL-21. It would be of interest to assess IL-21 production by Tfh cells in parallel to IL-21R expression in specimens from HIV-1 infected patients to understand whether these two parameters are interconnected. This point may be difficult to be addressed in the future as the majority of patients receive ART; in **paper I** IL-21R expression in ART treated patients returned to level similar to what found in the controls.

The cytokine IL-7 was shown to indirectly affect B cell activation and survival, as described in **paper II**. We demonstrated that IL-7 is able to upregulate CD70 expression on T cells; this event, in turn, through the triggering of CD27 receptor on B cells, led to proliferation of B cells that displayed a phenotype of differentiated cells and secreted high levels of Igs. These effects were abrogated by CD70 blocking experiments. In addition, IL-7 was shown to increase BAFF production by T cells, which enhanced B cell survival; when blocking BAFF signaling, B cell apoptosis increased. Our data suggest that IL-7 can enhance the B cell stimulatory potential of resting T cells via the upregulation of CD70, possibly contributing to a generalized B cell activation in conditions associated with elevated IL-7 levels, including HIV-1 infection.

The results of **paper II** are surprising and important as we showed that upregulation of CD70, induced by IL-7, leads to B cell activation and immunoglobulin production in absence of any specific antigen. It is possible that in diseases accompanied by an elevated IL-7 production, as SLE and HIV-1 infection, augmented CD70 expression on T cells may lead to a generalized activation of B cells independently of their specificity. This observation highlights similarities between the pathogenesis of SLE and dysregulated B cell responses during HIV-1 infection.

Paper II also highlighted the possibility that activated T cells may participate in vivo to BAFF production, a property previously assigned to BM stromal cells and APCs.

I continued by studying whether the mechanism presented in paper II could account for some of the B cell dysfunctions, including hyperactivation, described to take place during HIV-1 infection [155]. In **paper III,** we found an increased expression of CD70 on CD4+ T cells in correlation with CD4+ T cell depletion or viremia in HIV-1 infected patients. Surprisingly, we could not detect any correlation between plasma IL-7 levels and CD70 expression, suggesting that the CD70 upregulation in CD4+ T cell depleted patients may be independent of IL-7. In earlier work [168], it was shown that high level of IL-7 during HIV-1 infection inversely correlated with CD4+ T cell counts. While ART leads to an increased CD4+ T cell number, it is not clear whether high IL-7 production takes place in lymphoid tissues of treated patients; on the contrary it has been shown that production of IL-7 from lymphoid tissue may be permanently affected, due to collagen deposition which begins at the early phase of infection [136]. Based on their chemokine profile, we could show that CD4+ CD70+ T cells produce ex-vivo pro-inflammatory cytokines and have the potential to migrate to sites of inflammation. In addition, a putative role for CD70+ CD4+ T cells in providing bystander help for B cell activation was suggested in **paper III** by the association between the increased frequency of CD4+ CD70+ T cells and higher CD38 and CD95 expression on memory B cells, as well as increased B cell proliferation and plasma IgG levels in HIV-1 infected individuals. These results highlight the possible role of peripheral CD4+ CD70+ T cells in providing B cell stimulatory signals which may contribute to increased B cell activation, a characteristic of HIV-1 infection.

It would have been of interest to expand this study by further characterizing the properties of CD70+CD4+ T cells and by asking whether these cells express markers of immune activation, typically found during HIV-1 infection. The measurements of BAFF expression in T cells and soluble BAFF in plasma would also lend support to the mechanism presented in **paper II** which leads to B cell activation. A relevant aspect to study further is whether IL-7 is still produced in the LTs of HIV-1 infected patients to a higher level than what can be found in healthy controls. This latter study could clarify the factors leading to CD70 up-regulation on CD4+ T cells during HIV-1 infection. As previously mentioned it is difficult to conduct studies on viremic HIV-1 patients as the majority of patients in the clinics in Stockholm receive ART and become aviremic. However as we found that a higher frequency of CD70+CD4+ T cells was found in the blood of patients who remained significantly lymphopenic in spite of ART for several years, these studies could be conducted in a few individuals of lymphopenic patients.

In **paper IV,** we show that ART initiation during PHI did not prevent phenotypical changes of T cells, which were comparable with the dysfunctional phenotype identified in HIV-1 infected patients starting treatment during the chronic phase of infection. The major phenotypical changes identified were related to increased immune activation (HLA-DR+ and CD38++ mainly on CD4+ T cells) and the down-regulation of CD127 (on CD8+ T cells and subpopulations). In contrast to the dysfunctional phenotypes identified in both groups of HIV-1 infected patients, the number of HIV-1 DNA copies found in blood of EA patients was significantly lower than what was measured in LA patients. It cannot be excluded that despite successful ART, the size of the virus reservoir may be different in relevant lymphoid tissues where some virus replication may still take place [206], accompanied by abnormal immune activation. A clear limitation to this study presented in Paper IV is that the EA and LA patients were not followed from the time of ART initiation; accordingly we do know about the size of virus reservoir at ART initiation in these EA and LA patients. In addition we have not studied if the levels of immune activation were comparable in both groups at the time of ART initiation.

We measured several soluble parameters of inflammation in blood (Paper IV) and found that the levels of these molecules did not distinguish between EA and LA patients; in general the levels of these inflammation parameters did not distinguish between HIV-1 infected and controls. It was a real surprise to find elevated levels of BAFF in the serum of HIV-1 infected individuals, both EA and LA, compared to controls (**preliminary results**) and that BAFF levels correlated in the whole group of patients and controls to memory B cell populations which frequency is dysregulated during HIV-1 infection. The role of BAFF in affecting the biology of B cell sub-populations during HIV-1 should be further studied.

In recent years an increased number of HIV-1 infected patients have got access to ART in low- and middle-income countries [4]. HIV-1 infection and its treatment are associated with a series of biological events (e.g. inflammation, immune dysfunction), clinical factors (e.g. polypharmacy, multi-morbidity) and social factors (stigmatization) all influencing aging; the population of aging ART-treated individuals will therefore confront significant challenges [89, 207, 208]. The impact that the chronic low-level of immune activation will have on the immunological system of ART treated adults, now expected to live for decades, is not known [204]. The recently published START study [36] showed many health benefits in patients initiating ART during the asymptomatic phase of HIV-1 infection. The VISCONTI cohort study [212] is a good example of the benefits of early treatment initiation and how that can result in more individuals becoming post-treatment controllers for a variable period of time when interrupting ART.

These findings, in combination with the escalating cost for treatment in countries with high number of HIV-1 infected individuals, has promoted scientists operating in the HIV-1 field to investigate novel strategies for a HIV-1 cure [223]. Clearly, a cure strategy would both benefit infected individuals and reduce the economic burden for affected countries [224]. It should be emphasized that there is an urgent need to identify biomarkers to characterize immune-preservation in patients initiating ART during PHI; these may help to pin-point patients who may be taken off from ART and be included in future studies for cure intervention.

7 MATERIALS AND METHODS

Study populations

The patient material included in papers I, III and IV and material from HIV-1 uninfected donors consisted of blood samples collected in collaboration with the HIV-1 clinic Venhälsan at Stockholm South General Hospital, Sweden.

Paper I

Peripheral blood was collected from healthy subjects (n=23) and HIV-1 infected patients (n=40). Among HIV-1 infected patients, 20 were undergoing ART and started treatment in the chronic phase of infection [mean duration of infection: 13.5 (1.5-25) years; duration of treatment: 8.15 (1.5-17) years, CD4+ T cell count: 619 (222-1412) cells/ml and HIV-1 RNA <50 copies/ml] and 20 were naïve to treatment [mean duration of infection: 5.4 (1.5-12.5) years; CD4+ T cell count: 600 (278-1357) cells/ml and HIV-1 RNA 33960 (233- 132 000 copies/ml].

Paper II

Peripheral blood mononuclear cells (PBMCs) were separated from buffy coats collected from healthy blood donors at the Karolinska University Hospital, Solna, Sweden.

Paper III

Blood samples were obtained from healthy donors (n=10) and HIV-1 infected patients (n=55) divided into three different cohorts; 20 patients were naïve to treatment [CD4+ T cell counts higher than 350 cells/μl (mean = 600 ± 271 cells/ μl) and HIV-1 RNA 21500 (200-132000)], 25 patients received ART [CD4+ T cell counts higher than 200 cells/μl (mean 497 ± 222 cells/ μl and HIV-1 RNA < 25 copies/ml] and the third group consisted of ten CD4+ T cell lymphopenic patients undergoing ART [CD4+ T cell counts lower than 200 cells/μl (mean = 145 ± 65 cells/ μl) for a period of 3-20 years and HIV-1 RNA <100 copies/ml].

Paper IV and preliminary data

Blood samples were collected from healthy controls (n= 25) and HIV-1 infected patients (n=34). Amongst the HIV-1 infected patients, 17 patients started ART treatment during PHI [median duration of treatment: 25 (7-59) months, CD4+ T cell count: 840 (390-1340) cells/ml and HIV-1 RNA <50 copies/ml], and 17 patients started treatment in the chronic phase of infection [median

duration of treatment: 29 (12-60) months, CD4+ T cell count: 780 (250-1000) cells/ml and HIV-1 RNA <50 copies/ml].

Isolation of PBMCs from HIV-1 infected patients and healthy controls (all papers)

A high-density centrifugation technique was used in order to isolate mononuclear white blood cells from whole blood. 15 ml of sodium chloride solution containing high molecular weight sucrose-polymers was poured into a 50 ml tube. The blood sample was then carefully applied on top of the solution, and the tube centrifuged without brake for 20 minutes at 2000 rotations per minute (rpm). The layer of mononuclear cells was then transferred to a new tube, with the help of a pipette. The cells were washed a few times in Phosphate Buffer Solution (PBS) and once in Roswell Park Memorial Institute (RPMI) medium. The RPMI medium used in all assays included L-Glutamine, Sodium pyruvate, HEPES buffer and high glucose and completed with 10% fetal calf serum (FCS) and 1% penicillin- streptavidin-fungizone solution. PBMCs were cryoperserved in FCS with 10% dimethylsulphoxide (DMSO) in liquid nitrogen (-196°C) until further analyses were conducted.

Cell culture

In vitro B cell activation through BCR and TLRs (paper I)

PBMCs and purified B cells from healthy donors were cultured in RPMI medium in the presence of different TLR agonists and anti-BCR for 24 hours.

Co-culture of IL-7 pre-treated peripheral T cells and B cells (paper II)

T cells and B cells were separated from PBMCs with microbeads using Pan T cell Isolation Kit and B cells isolation kit II respectively. Purified cells were cultured in RPMI medium and pre-treated with recombinant IL-7 for 5 days. For T cells -B cells co-culture, a ratio of 1:1 T cells-B cells was used.

In vitro CD70 upregulation with different stimuli associated with chronic HIV-1 infection (paper III)

PBMCs of non-infected individuals and ART-treated non-lymphopenic patients were stimulated with a HIV-1 strain, inflammatory cytokines, γ-chain cytokines and a T cell receptor cross-linking antibody for 5 days.

Flow cytometry (all papers)

Flow cytometry is a method that enables multiple and quantitative evaluation of millions of heterogeneous immune cells based on light scattering and fluorescence. In a flow cytometer, the high flow generates a single-cell stream of cells that passes laser beams. The size of each cell is predicted by how the light is scattered before quantification by a front filter (forward scatter), and the light scatter on a side filter (side scatter) measures the cell granularity. The laser induces excitation of fluorescent parts on the cells, and the emitted light is captured and measured. Antibodies conjugated to fluorescent molecules attach to specific proteins on the cell surface and therefore information about several markers of interest can be detected on each cell.

Enzyme-linked immunosorbent assay (ELISA) (all papers)

ELISA is a method for quantification of cytokines and other analytes in solution. A specific antibody was used to coat a microtiter plate. After addition of the sample, the specific antibody on the plate will capture the protein of interest, while a second antibody, which is used for detection, binds a different epitope on the same protein. The detection antibody is labeled with biotin, which allows subsequent binding of a streptavidin-conjugated enzyme. Any unbound reagent is removed by washing. After addition of a substrate, a color reaction develops that is directly proportional to the amount of protein bound. The concentration of the protein in the sample is determined by comparison with a standard curve of known protein concentrations.

To quantify antibodies in plasma or serum, plates were coated with the selected antigen. Binding of antibodies to the selected antigen was detected by a secondary anti-Ig detection antibody, directed towards the immunoglobulin type of interest. In the papers included in this thesis, IgA, IgM and IgG were detected.

HIV-1 infection of humanized mice (paper III)

Humanized NOD scid gamma (NSG) mice, are a strain of inbred laboratory mice that lack mature lymphocytes. When 4-6 weeks old, the mice were conditioned with two intraperitoneal injections of Busulfan, which is an alkylating agent used to inhibit the reproduction and growth of white blood cells, followed by an injection of freshly isolated human cord blood CD34+ hematopoietic stem cells. Mice were screened for cell engraftment by monitoring human CD45 expression in the peripheral blood. The humanized mice were after 174 up to 195 days of transplantation infected with HIV-1 (strain BaL-1, 10 000 $TCID_{50}$) by intravenous injection. Three months post-infection, the mice were anesthetized through an intraperitoneal injection. Blood samples were collected via cardiac puncture and cervical dislocation was used for secondary euthanasia. VL was measured from plasma; lymph nodes and bone marrow were dissociated with syringes and passed through a nylon strainer to obtain single-cell suspensions for flow cytometry.

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

PBMC DNA was obtained by manual extraction. Total PBMC HIV-1 DNA was quantified using a homemade Taqman real-time assay with primers located in conserved regions of the the HIV-1 genome. To correct for minor deviations from the expected DNA input, total HIV-1 DNA copy numbers were normalized on a beta-globin standard curve and expressed as copies per million PBMCs. To ensure accurate normalization, HIV-1 and beta-globin DNA were amplified in the same reaction tube. The standard curve for quantification of total HIV-1 DNA was obtained from serial dilutions of the pNL4-3 plasmid containing the full HIV-1 genome. The proviral DNA copy number, combined with the total number of cells present, can give an estimate of the frequency of cells harboring HIV-1 DNA. The limit of detection was 10 HIV-1 DNA copies per million PBMCs.

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