Chronic immune activation and inflammation in HIV-infected immunological non-responders

A study of innate and adaptive immunity and effect of probiotic intervention

by

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Abbreviations

AIDS	acquired immunodeficiency syndrome
APC	antigen-presenting cell
ART	antiretroviral therapy
AT-2 HIV	aldrithiol-2-inactivated HIV
aTreg	activated regulatory T cell
CCR5	C-C chemokine receptor 5
CD4 count	$CD4^+$ T cell count
CM	central memory
CMV	cytomegalovirus
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte antigen
CXCR4	C-X-C chemokine receptor type 4
DC	dendritic cell
ELISA	enzyme-linked immunosorbent assay
EM	effector memory
FMO	fluorescence minus one
FMT	fecal microbial transplantation
GALT	gut-associated lymphoid tissue
HBV	hepatitis B
HCV	hepatitis C
HIV	human immunodeficiency virus
(hs)-CRP	(high sensitivity) C-reactive protein
IDO	indoleamine 2,3 dioxygenase
IFN	interferon
IL	interleukin
INR	immunological non-responder
IP-10	interferon-inducible protein 10
IR	immune responder
KTR	kynurenine/tryptophan
LC-MS	liquid chromatography-tandem mass spectrometry
LPS	lipopolysaccharide
MAIT	mucosal-associated invariant T cells
mDC	myeloid dendritic cell
MSM	men who have sex with men
NK	natural killer cell
PBMC	peripheral blood mononuclear cells
PLWH	people living with HIV
PD-1	programmed death 1
pDC	plasmacytoid dendritic cell
rTreg	resting regulatory T cell
Treg	regulatory T cell
SIV	simian immunodeficiency virus
SLAMF7	Signaling lymphocytic activation molecule family member 7
SNAE	serious non-AIDS events (cardiovascular disease, cancer, kidney disease, liver disease, osteopenia or osteoporosis, and neurocognitive disease)
TBC	tuberculosis
TF	tissue factor
Th	T helper
TLR	toll like receptor
TNFα	tumor necrosis factor
rh	recombinant

List of publications

Paper I: **Reduced levels of D-dimer and changes in gut microbiota composition after probiotic intervention in HIV-infected individuals on stable ART**

Birgitte Stiksrud, Piotr Nowak, Felix Nwosu, Dag Kvale, Anders Thalme, Anders Sonnerborg, Per M. Ueland, Kristian Holm, Stein-Erik Birkeland, Anders E. A. Dahm, Per M. Sandset, Knut Rudi, Johannes R. Hov, Anne M. Dyrhol-Riise and Marius Trøseid

J Acquir Immune Defic Syndr. 2015;70:329-337

Paper II: **Plasma IP-10 is increased in immunological non-responders and associated with activated regulatory T cells and persisting low CD4 counts**

Birgitte Stiksrud, Kristina Berg Lorvik, Dag Kvale, Tom Eirik Mollnes, Per Magne Ueland, Marius Trøseid, Kjetil Taskén and
Anne M. Dyrhol-Riise

J Acquir Immune Defic Syndr. 2016;73:138-148

Paper III: **Activated dendritic cells and monocytes in HIV immunological non-responders; HIV-induced IP-10 correlates with low future CD4 recovery**

Birgitte Stiksrud, Hans C. D. Aass, Kristina B. Lorvik, Thor Ueland, Marius Trøseid and Anne M. Dyrhol-Riise

AIDS. 2019;33:1117-11293

Summary

Human immunodeficiency virus (HIV) still remains a significant threat to global health, particularly in low- and middle-income countries, although antiretroviral therapy (ART) has dramatically improved the prognosis for people living with HIV (PLWH) and is recommended to all PLWH.

Despite improved HIV care and access to ART, significant gaps in life expectancies persist between PLWH and the general population, partly because of their increased risk of non-AIDS comorbidities such as cardiovascular disease, cancer, renal dysfunction and osteoporosis. Extensive research supports a strong association between low-grade inflammation and the increased risk of developing non-AIDS comorbidities in treated HIV infection. Alterations in the gut microbiota and destruction of the gut mucosal barrier with subsequent leakage of microbial products into the systemic circulation, so-called microbial translocation, have been shown to contribute to this inflammation. Therapeutic manipulation of factors driving the inflammation in order to reduce the chronic inflammatory state can potentially improve the prognosis in PLWH on ART, and is currently subject to numerous intervention studies worldwide.

Approximately 12-30% of PLWH on ART do not normalize their $CD4^+$ T cell count (CD4 count), denoted immunological non-responders (INR). As INR constitute a group of patients with poorer prognosis, research aiming to investigate changes in the innate and adaptive immune system in these patients is of particular relevance. Furthermore, identification of potential biomarkers associated with poor $CD4^+$ T cell recovery might contribute to the understanding of immune pathways associated with INR status and identify targets for intervention. It is widely documented that advanced HIV disease with a low pre-ART CD4 count reduces the probability of achieving a normal CD4 count on ART. In fact, a substantial proportion of PLWP still enters HIV care and start ART as late presenters with advanced immune deficiency in both high and low income countries, interpreting INR as a clinical relevant patient group for the future.

In this thesis we characterized differences in chronic immune activation and inflammation between a cohort of INR and a cohort of ART-treated PLWH with

adequate CD4 recovery and searched for biomarkers for inadequate immunologic response to ART (**paper II** and **III)**. In a randomized controlled pilot study we explored the safety and the effect of probiotic intervention upon microbial translocation and chronic inflammation in PLWH on ART with a subnormal CD4 count (**paper I)**. The patients were recruited from the outpatient clinic at Department of Infectious Diseases at Oslo University Hospital with contribution from Karolinska University Hospital in **paper I**.

The study in **paper I** was one of the first to target the gut microbiota and to analyze deep sequencing data in PLWH on ART with a subnormal CD4 count. We found a significant reduction in plasma levels of D-dimer and a tendency to reduced levels of CRP and interleukin-6, as well as increased fractions of *Bifidobacteria* and *Lactobacilli* in fecal samples after eight weeks of intervention with a multistrain probiotic, indicating that probiotic intervention might have a beneficial effect upon inflammation in these patients. However, recent small studies have not reported similar effects of probiotics, and larger studies with long-term follow-up are needed to conclude.

In **paper II** and **III** we showed that the INR cohort displayed a more activated innate and adaptive immune system compared with PLWH on ART with normalized CD4 counts. In INR, the high frequencies of activated regulatory T cells were associated with reduced HIV-specific responses *in vitro*. Plasma interferon-inducible protein 10 (IP-10) was increased in the INR cohort and together with kynurenine/tryptophan ratio negatively correlated with future CD4 recovery. In depth studies discovered that the IP-10 increase in monocytes induced by *in vitro* HIV-stimulation, was associated with reduced CD4 gain after both two and four years.

These findings contribute to the understanding of the immunological mechanisms causing an inadequate CD4 recovery in a group of INR, and suggest IP-10 as a potential biomarker for incomplete restoration of the CD4 count, although the usefulness in clinical practice has to be further elaborated in larger prospective studies.

1. Introduction

1.1 History and epidemiology

1.1.1 The origin of HIV

A report on five previously healthy homosexual men in Los Angeles with fatal acquired T cell defects and pneumocystis carinii, cytomegalovirus (CMV) and mucosal candida infections [1, 2], marked the start of the human immunodeficiency virus (HIV) pandemic in 1981. Within two years scientist isolated the retrovirus lymphoadenopathy-associated virus (LAV), a virus related to the human T cell leukemia virus (HTLV), as the causative agent for the acquired immunodeficiency syndrome (AIDS) [3, 4]. In 1986, the virus changed name to HIV [5], or more correctly HIV-1, as HIV-2 was isolated in West-Africa the same year [6]. HIV-2-infection progresses more slowly to AIDS, is less transmissible and is still largely confined to West-Africa [7].

On the other hand, HIV-1 spread dramatically, initially within men who have sex with men (MSM), intravenous drug abusers (IDU) and people who had received blood transfusions [8], but was in 1984 also identified among heterosexuals in Central Africa [9, 10]. Later on, phylogenetic analyses indicate that HIV-1 originated in Central Africa, probably after transmission from apes to humans in the beginning of the 20th century [11]. The first human HIV-1 infection was confirmed retrospectively in samples from an adult male from Kinshasa, Democratic Republic of Congo in 1959 [12]. In Europe, AIDS was diagnosed retrospectively in a Norwegian sailor and his family members, with symptom onsets already in the late 1960s [13].

HIV-1 is classified into four groups, representing separate cross-species transmissions, three from chimpanzees (M-major, N-non-M, non-O and O-outlier) and one from gorillas (P) [11]. HIV-1 group M consists of several genetically distinct subtypes (A, B, C, etc), is responsible for the pandemic spread [11] and will be subject for this thesis, hereafter termed HIV. Subtype B is the most prevalent worldwide and predominates in

Africa and India, whereas subtype C predominates in Europe, America and Australia [7]. The other groups (N, O and P) are largely restricted to Central and West-Africa and have minor global significance [11].

1.1.2 Epidemiology

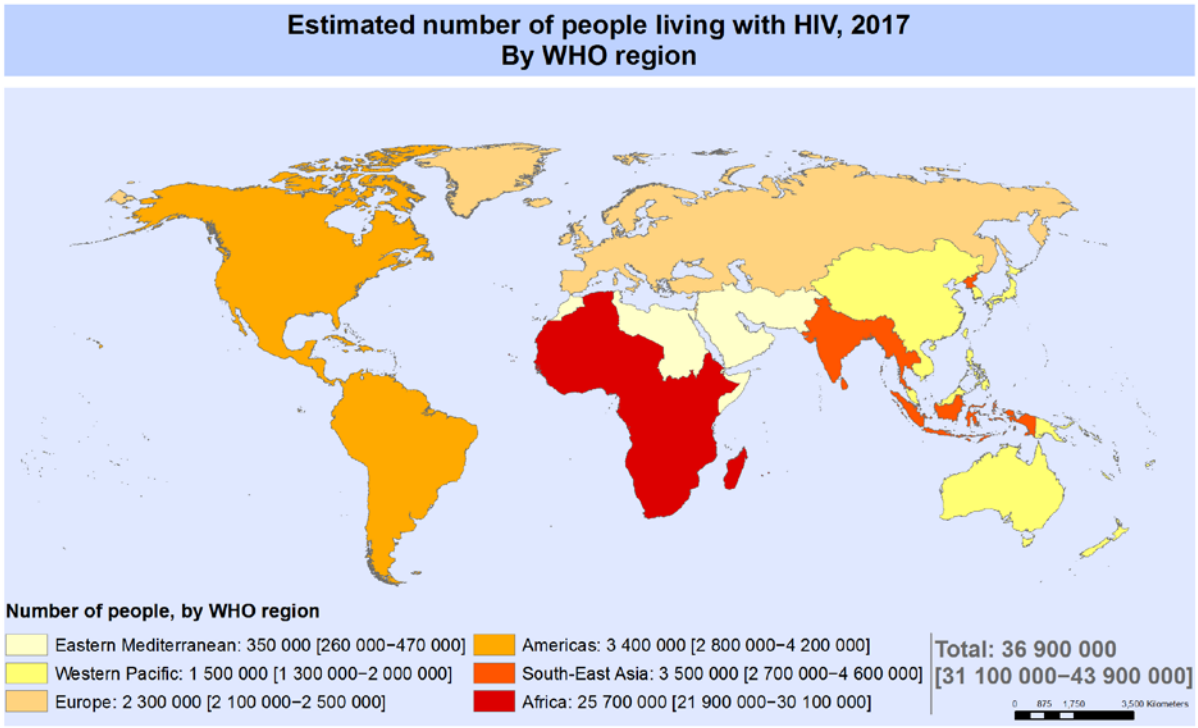


Figure 1. Estimated global HIV prevalence by WHO region, in 2017. Modified from WHO.

Since the start of the HIV epidemic in the early 1980's, UNAIDS estimates that 77.3 million people have become infected with HIV worldwide and 35.4 have died from AIDS-related illnesses [14]. By the end of 2017, 36.9 million people were living with HIV, the Sub-Saharan Africa still being the most severely affected region accounting for over two-thirds of the PLWH population [14]. The enormous investment in the HIV response over the past years are paying off and new HIV infections have been reduced by 47% since the peak in 1996, from 3.4 million to 1.8 million in 2017, mainly driven by the decline in incidence in Sub-Saharan countries. 940 000 died from AIDS-related illnesses in 2017, a reduction of 51% since 2004 (1.9 million). However, some alarming trends do exist. In Eastern Europe and Central Asia the numbers of new infections and deaths still continue to rise [14]. The people at risk differ between regions. In Eastern

and Southern Africa young women have the highest risk of HIV infection. Conversely, key populations such as MSM, IDU, female sex workers, transgender women and their sexual partners accounted for 95% of new HIV infections in Eastern Europe, Central Asia, the Middle East and North Africa [14].

In Norway the prevalence of PLWH by the end of 2018 was 6468 persons, two thirds of them were men [15]. In total, 52.8% were reported to be transmitted heterosexually, 33.2% homosexually and 10% by intravenous drug use [15]. The incidence of new HIV infections has mainly decreased over the ten last years; from 299 cases in the peak year 2008 to 191 cases in 2018. In 2018, more than 80% were infected abroad, 58% were immigrants infected before arriving Norway [15].

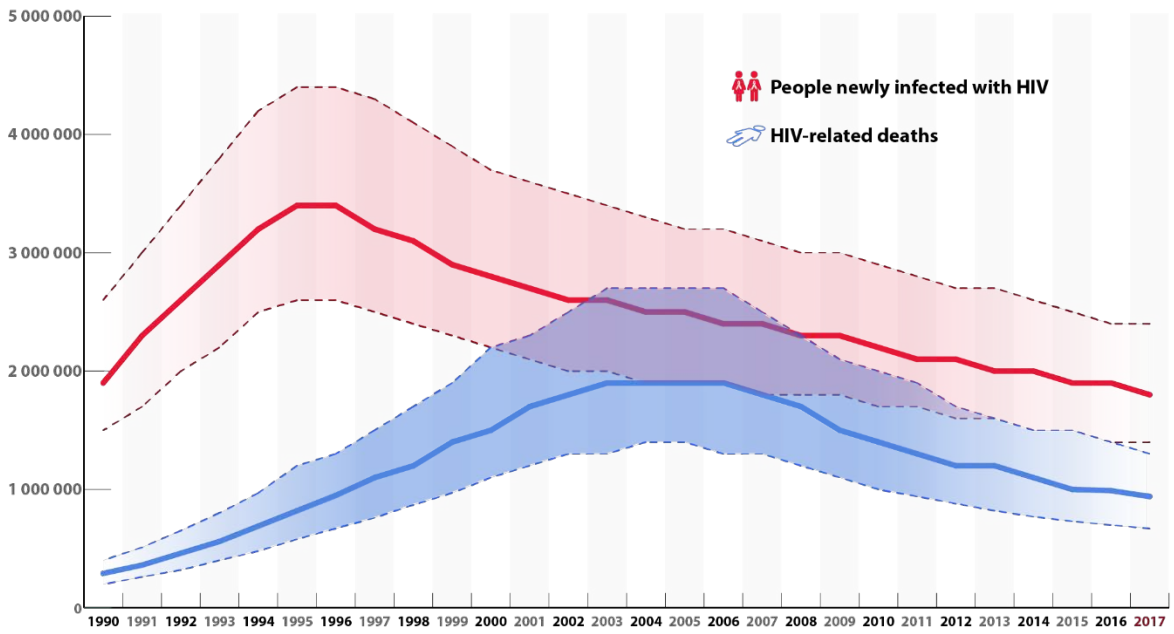


Figure 2. Decline in HIV incidence and mortality over time. (WHO)

In 2014, the UNAIDS launched the 90-90-90 targets aiming to end the AIDS epidemic by 2030: “By 2020, 90% of all PLWH will know their HIV status, 90% of all people with diagnosed HIV infection will receive sustained (antiretroviral therapy) ART and 90% of all people receiving ART will have viral suppression” [16]. This has led to a global scale-up of HIV testing and access to ART and HIV care. In 2017, 75% of PLWP

knew their status. Among those, 79% were accessing treatment and 81% of people receiving ART were virally suppressed [14]. In other terms, by the end of 2017, 59% of PLWH were accessing ART, but with large geographic variations ranging from 29% in the middle East and North Africa to 36% in Eastern Europe and Central Asia and 78% in Western and Central Europe and North America [14].

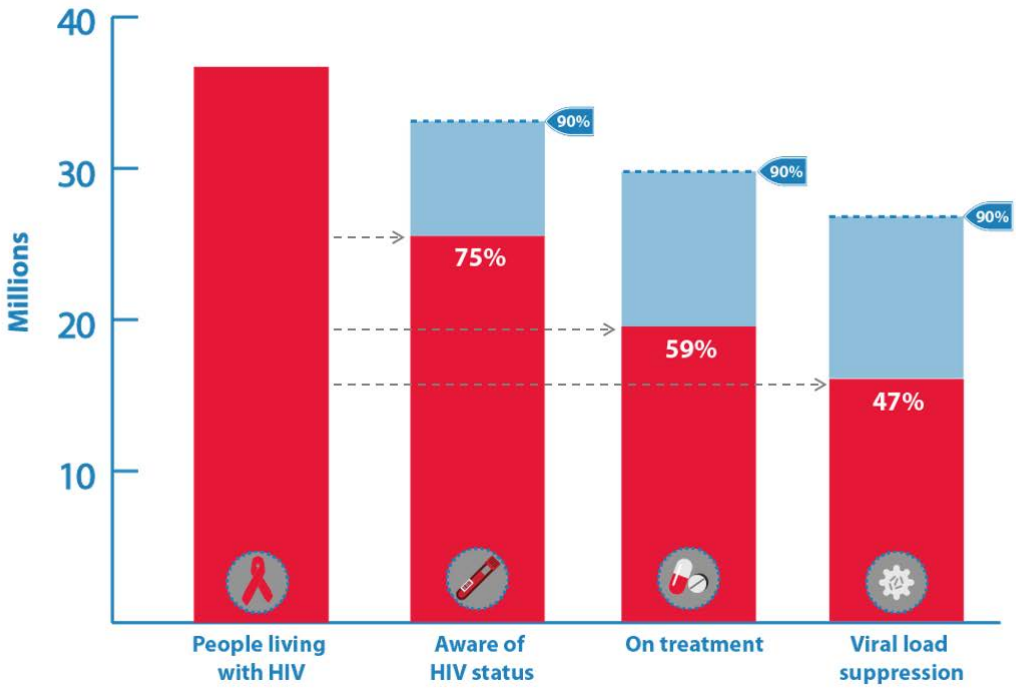


Figure 3. Progress towards 90-90-90 target, 2017. Modified from WHO

1.1.3 Transmission

Most of the PLWH worldwide have been transmitted through sexual contact, although the risk is very low, estimated to less than 0.1% by a vaginal intercourse and about ten times higher for anal intercourse [17]. HIV is also transmitted through blood, previously by blood transfusions with untested blood products, by shearing of infectious needles by intravenous drug abusers and vertically from mother-to-child during pregnancy or by breastfeeding [17]. High viral load as present during acute HIV infection, increases the risk of transmission substantially. Furthermore, coincident sexually transmitted infections, genital ulcers and intimate partner violence also enhance HIV sexual

transmission, whereas male circumcision and condom use are shown to reduce sexual transmission risk [7]. ART as prevention is extremely effective and the recently published PARTNER1 and PARTNER2 studies support the message "undetectable equals untransmittable" [18, 19]. No linked within-couple transmissions were observed through condomless sex in serodiscordant heterosexual [18] or gay couples [19] when the HIV positive partner was virally suppressed on ART. Similar findings were reported from the 052 HPTN study in 2015; ART to serodiscordant couples was associated with a 93% lower risk of linked partner infections and no HIV transmission occurred when the HIV-infected partner was virally suppressed [20]. In several countries, pre-exposure prophylaxis (PrEP) with antiretroviral drugs is now offered to individuals known to be at high risk of becoming infected with HIV [21].

1.2 Virology

1.2.1 Structure and genome

HIV is a single-stranded RNA lentivirus belonging to the *retroviridae* family [22]. Anchored in the lipid membrane are HIV envelope spikes comprising three external gp120 and three transmembrane gp41 subunits [23]. Inside the virion lies the outer p17 matrix protein layer, and the cone-shaped inner capsid composed of the p24 protein that surrounds the virion core. Apart from to copies of positive-sensed single-stranded RNA, the core houses the nucleocapsid protein p7 and the viral enzymes reverse transcriptase and integrase [22].

The HIV genome consists of nine genes flanked by 5' and 3' long terminal repeats that are important for transcription initiation [24]. The structural gene *env* codes for the envelope glycoproteins, *gag* for the capsid proteins p17, p24 and p7 and *pol* for the viral enzymes reverse transcriptase, integrase, protease and RNAse [24]. The *tat* and *rev* genes encode viral regulatory proteins and the remaining genes *vif, vpr, vpu* and *nef* code for viral accessory proteins [24].

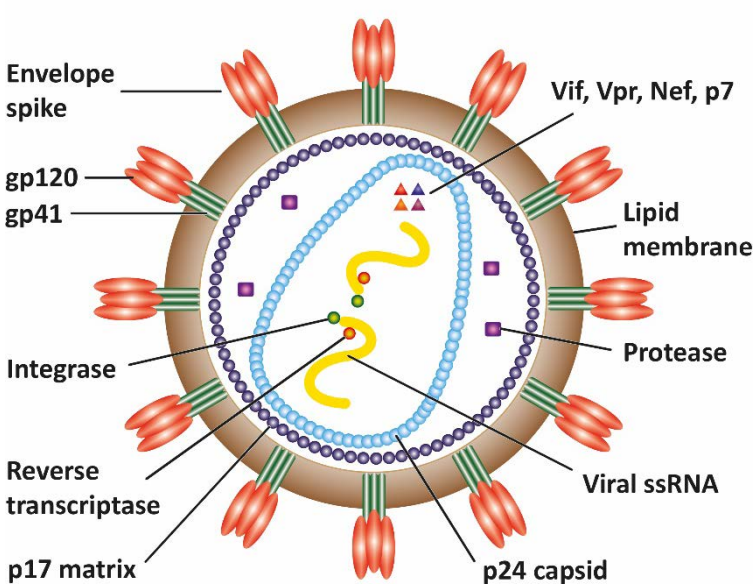


Figure 4. HIV structure. Modified from niaid.

1.2.2 Replication cycle

The main target of HIV is activated effector memory (EM) CCR5 expressing $CD4^+$ T cells. After the initial binding of gp120 in the envelope spike to the surface CD4 receptor, the envelope complex undergoes a conformational change, exposing the binding site for a second cellular receptor, usually the chemokine receptors CCR5 or CXCR4 (step 1). This allows for insertion of gp41 into the cell membrane, initiating the fusion and release of the viral core into the cell cytoplasm. The viral capsid dissolves gradually, viral single-stranded RNA is reverse transcribed to DNA by the viral enzyme reverse transcriptase (step 2) and converted to double-stranded DNA, forming a pre-integration complex that is imported into the nucleus. This reverse transcriptase process is highly prone to errors, and together with the high-level replication rate, cause considerable genetic variation of new viral strains [25]. Viral integrase cleaves and transfers the viral DNA into the host genome, a process completed by host enzymes, forming a stable provirus (step 3) [26]. The cell is now permanently infected.

With help from the viral proteins Rev and Tat, host enzymes transcribe HIV DNA into mRNAs. After exportation to the cytoplasm these mRNAs are translated into viral proteins as parts of the Gag precursor polypeptide (step 5), which assembles the viral

particles at the plasma membrane. Finally, the retrovirus buds off the host cell and HIV proteases cleave the Gag and Gag-Pol precursor polypeptides into structural components and viral enzymes creating a mature and infectious new virus (step 6) [26].

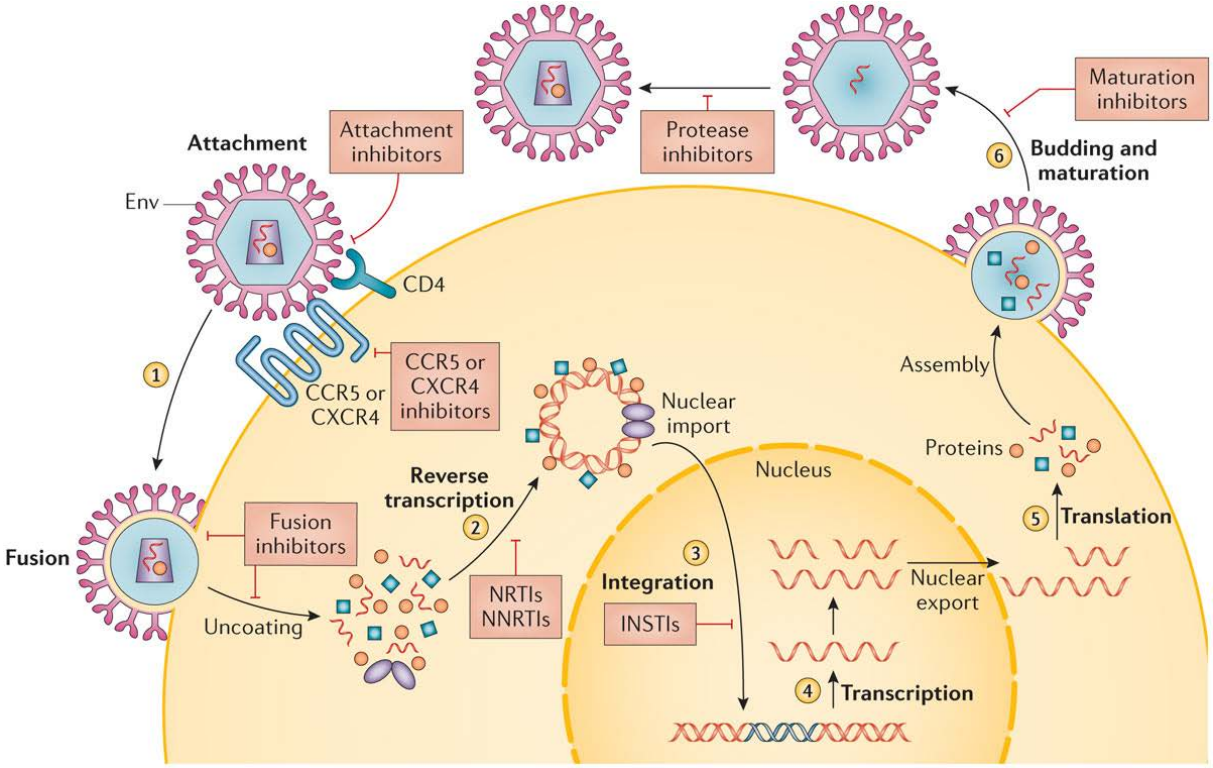


Figure 5. The HIV life cycle. NRTIs, Nucleoside Reverse Transcriptase Inhibitors; NNRTIs, Non-Nucleoside Reverse Transcriptase Inhibitors; INSTs, Integrase Strand Transfer Inhibitor.

1.3 Clinical characteristics of untreated HIV infection

The clinical course of HIV-infection can be divided in three phases [27]. During the acute infection, 2-6 weeks after transmission when the viremia peaks, 40-90% experience flu-like symptoms with fever, fatigue, lymphadenopathy, myalgia, pharyngitis, a maculopapular rash, anorexia and diarrhea [28]. The symptoms last for about two weeks, but can persist for a longer time [28].

The viremia eventually decays, reaching a set point after about 3-6 months, marking the transition to the chronic phase also called clinical latency [27]. The plasma HIV RNA level is relatively stable, although the number of $CD4^+$ T cells after some time begins to decrease [27]. During this period the patients usually have none or mild symptoms such as lymphadenopathy and gastrointestinal discomfort, and some experience fatigue [27]. As the $CD4^+$ T cell count (CD4 count) declines oropharyngeal and vulvovaginal candidiasis, recurrent pulmonary infections as well as herpes zoster infections may become frequent [29]. This chronic phase usually lasts for 7-10 years, however, the duration is highly variable [27]. Some PLWH control the infection for decades and do not develop AIDS [29, 30] and a few percentages maintain low or undetectable levels of HIV RNA in absence of ART, so-called HIV- or elite controllers [31].

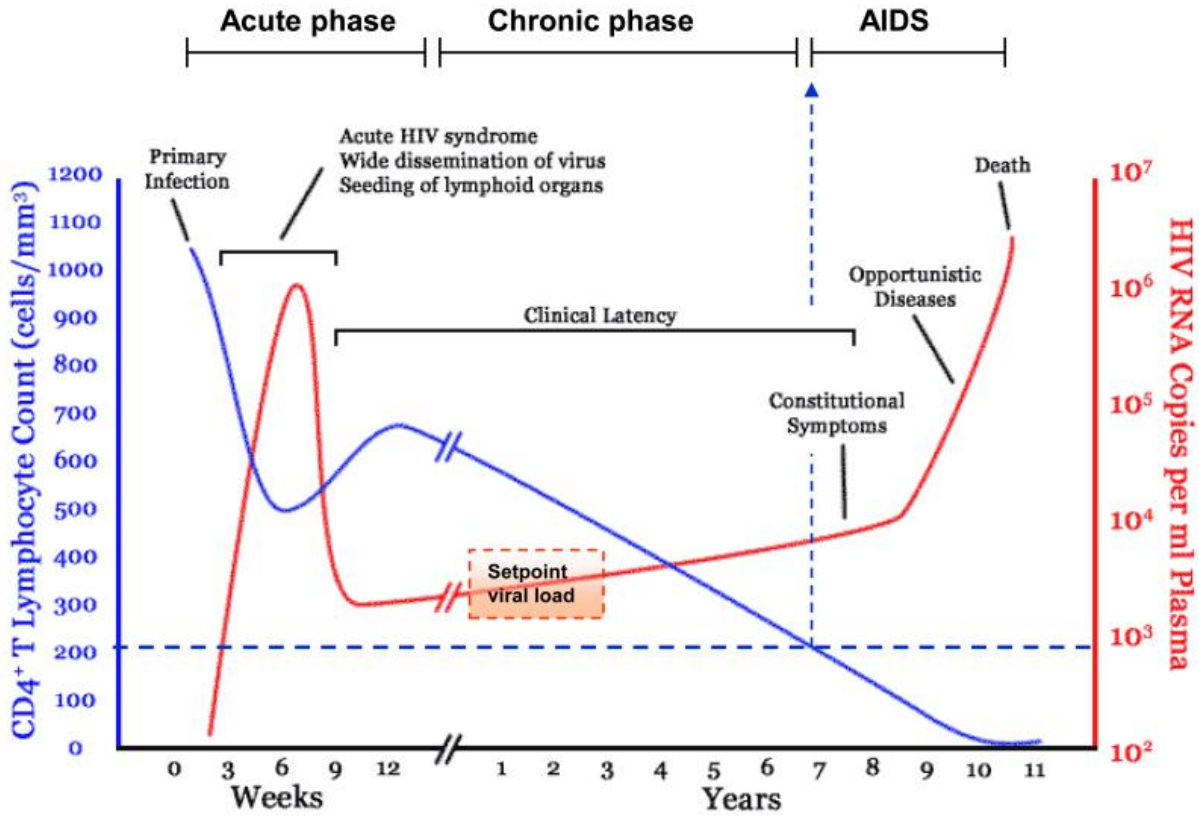


Figure 6. Natural course of untreated HIV infection. Following the acute infection when HIV viremia peaks and several persons experience flu-like symptoms, the plasma HIV RNA load decreases, reaching a steady state level and the infection enters a chronic phase with few symptoms. Immune control is gradually lost and the patients develop AIDS, usually several years after seroconversion.

The late stage of HIV disease with AIDS development is characterized by drop of CD4 count to less than 200 cells/µL, concomitant raise in viremia and presentation of opportunistic diseases and malignancies [27]. Without ART, median survival with AIDS is less than two years [32].

1.4 Antiretroviral therapy (ART)

Lifelong antiretroviral therapy has revolutionized the life-expectancies for PLWH and changed the HIV infection from a lethal illness to a chronic disease. In 1987 treatment with the reverse transcriptase inhibitor azidothymidine was shown to reduce opportunistic infections and mortality in patients with AIDS [33], however viral resistance developed quickly [34]. The major breakthrough came a decade later in 1996, with the discovery of the protease inhibitors, which in combination with two nucleoside-analogue reverse transcriptase inhibitors, improved prognosis for PLWH substantially [35, 36]. This marked the beginning of the highly active antiretroviral therapy (HAART, later termed ART) era and since then, combination of three different antiretroviral drugs has been the gold standard in the treatment of PLWH. Over the years with development of less toxic drugs and increased knowledge of the therapeutic benefit, treatment guidelines have evolved from expectancy to universal treatment [37, 38]. In 2006, the SMART study showed that CD4 count guided interrupted ART was associated with increased morbidity and mortality [39]. The START study demonstrated in 2015 the clinical benefit of immediate ART in PLWH with CD4 T cells count > 500 cells/µL vs deferral to CD4 count < 350 cells/µL [40]. Based on these results together with the "treatment as prevention" principle [20], the World Health Organization (WHO) recommended ART to all PLWH, regardless of WHO clinical stage and at any CD4 cell count, in 2016 [38].

ART targets different steps of the HIV replication circle (see Figure 5). To date lifelong commitment to ART is necessary to maintain viral suppression as ART does not eliminate non-replicating provirus, and consequently does not eradicate HIV. Side effects, interactions, development and transmission of drug resistance, social stigma,

adherence, incomplete restoration of the immune system are other limitations of ART. [21, 29]. Improvements such as individualized ART optimization and dual regimens are now included in treatment guidelines [41, 42] and long-acting drugs are in pipeline [21]. Providing ART to millions of people in high prevalent countries also constitutes a major burden on health systems. The search for a HIV cure, or at least at "functional" HIV cure that permanently controls HIV-infection, has been highly intensified and inspired by the report of the successfully cured HIV-infected patient – "the Berlin patient" a decade ago [43, 44]. Of note, a report of a "London patient" with 18 months remission was recently published [45]. These two patients were treated for blood cancer with allogeneic haematopoietic stem-cell transplantation using a donor homozygous for the delta-32 deletion of the CCR5 gene (HIV co-receptor), although "the Berlin patients" underwent a more extensive treatment.

1.5 Pathogenesis

1.5.1 Acute infection

Initial HIV-exposure is usually via the genital or gastrointestinal mucosa and new infections are established by one or a few genetic variants of CCR5 tropic virus [46]. The virus binds to susceptible $CD4^+$ T cells, but other cells expressing CD4 and chemokine receptors are also infected, including macrophages and dendritic cells (DCs) [47]. Myeloid (m)DCs transport the virus to a regional lymph node for presentation to $CD4^+$ T cells, causing activation and proliferation of these cells and systemic spread of the infection. HIV viremia can be detected in lymphoid tissue after two days and in blood around a week after transmission [47].

An intense viral replication in gut-associated lymphoid tissue (GALT) and other lymphoid tissues follows [30]. Plasma HIV RNA levels increase exponentially during the first weeks after HIV infection and reach a peak at 10^6-10^7 copies/mL after about 3-4 weeks [30]. The viremia is accompanied by a cytokine storm [48] and the degree of symptoms correlates with the viremia at this time-point [29]. A profound depletion of $CD4^+$ T cells occurs. However, HIV induces strong clonal HIV-specific cytotoxic $CD8^+$

T cells (CTL) responses, which destroy virally-infected cells and contribute to a partial over the infection, a fall in HIV RNA to a so-called set-point, combined with a gain in $CD4^+$ T cells [29, 30, 47]. The set-point level has prognostic significance for the future disease progression and is determined by complex virus-host factors [29, 49]. Neutralizing antibodies appear around three months after transmission and together with the CTL response, select for the emergence of viral escape mutations [30].

Most of the virus-infected $CD4^+$ T cells die. However, a small fraction develops into long-lived resting memory $CD4^+$ T cells [50]. These cells contain integrated HIV DNA and constitute the largest reservoir of HIV-infected $CD4^+$ T cells, mainly found in lymphoid tissue [51]. As long as they are resting there is no or minimal transcription of virus, but in case of activation of the cell, they can give rise to viremia [50].

1.5.2 Early infection in the GALT

During acute HIV infection the virus rapidly spreads throughout the GALT containing high numbers of HIV-susceptible CCR5 expressing activated effector EM $CD4^+$ T cells [52, 53]. As early as three days after transmission and prior to systemic viremia simian immunodeficiency virus (SIV) DNA is detected in GI tissues in SIV-infected Rhesus macaques [53] and in HIV-infected humans HIV DNA is identified in the gut in Fiebig I [54]. Hence, the gut viral reservoir is seeded within few days after transmission. In the course of the acute phase there is a massive loss of CCR5 expressing $CD4^+$ T cells in the gut lamina propria, mainly caused by direct viral cytopathic effects and bystander pyroptosis [55] and the depletion far exceeds what is observed in both peripheral blood and lymph nodes [52, 56-58]. Accompanied by the loss of CCR5 positive $CD4^+$ T cells, dysfunction of the gut mucosal immunity develops as there is a preferential depletion of T helper 17 cells (Th17) within this subset [59]. Furthermore, mucosal-associated invariant T (MAIT) cells [59, 60] and innate lymphoid cells 3 [61] seem to be reduced, although they are not directly infected by HIV. All these cell-types produce interleukin (IL)-17, and Th17 cells also secrete IL-21 and IL-22 that are crucial interleukins for maintaining gut immune defense and an intact gut barrier [59]. Whereas Th17 cells are preserved in SIV infection of the natural hosts Sooty mangabeys and African green monkeys [62], Th17 cells are depleted in pathogenic SIV infection in rhesus macaques

and HIV-infected humans already two-three weeks after transmission [62-65]. The loss of these cells is associated with mucosal damage, microbial translocation, chronic immune activation and disease progression in HIV- and SIV-infected subjects [59, 63, 65, 66].

These massive alterations in the epithelial barrier and gut immunity during the acute HIV infection have a major impact on disease progression and future health in PLWH. In 2006, Brenchley *et al* demonstrated that HIV-infected humans and SIV-infected Asian macaques had increased levels of lipopolysaccharide (LPS) in plasma, which correlated with activation of both the innate and adaptive system [67]. LPS is a component of the outer wall of gram negative bacteria and a potent activator of Toll like receptor (TLR) -4 [68]. Later on, numerous of studies have shown that the loss of gut mucosal integrity allows microbial products to leak into the gut mucosa and subsequently to the circulation contributing to chronic stimulation of the immune system, a process referred to as microbial translocation [66, 69-74].

1.5.3 Chronic immune activation and inflammation

A hallmark of HIV infection is the generalized activation of both the innate and adaptive immune systems, which is associated with a wide range of complications following HIV-disease [75-77]. Sooty mangabeys, the natural hosts for SIV infection remain healthy despite high SIV viremia and demonstrate minimal signs of immune activation. In contrast, the rhesus macaques show massive immune activation, loss of $CD4^+$ T cells and they develop AIDS following SIV infection [78]. This indicates that the depletion of $CD4^+$ T cells and disease progression are not caused by the direct consequences of HIV replication alone. Findings of a more closely relation between frequencies of activated $CD8^+$ T cells and mortality than virus burden and mortality in advanced HIV disease [79], low levels of immune activation in a small group of PLWH with high viremia and persistently stable CD4 count (virological non-progressors) [80] and correlation of immune activation and $CD4^+$ T cell loss in elite controllers [70], support this conclusion.

In untreated HIV infection T cell activation predicts disease progression, AIDS and infectious complications [79, 81, 82], while markers of innate immune activation, inflammation and coagulation are strongly associated with non-AIDS morbidity and mortality in treated HIV infection [75, 76, 83].

Immune activation in blood is well characterized, but lymphoid tissue is the major site for HIV-replication [84-86] and display higher fractions of activated T cells [86-88].

In untreated HIV infection HIV viremia is an important driver of immune activation [89, 90]. Levels of immune activation and inflammation correlate with viremia and are hence lower in HIV controllers [70, 79, 91, 92]. Still, in virally suppressed PLWP viral persistence can contribute [93-95], driving the immune activation both systemically [95, 96] and locally in lymph nodes and gut mucosal tissue [97, 98]. Also, the proportion of activated T cells highly exceeds the frequencies of infected $CD4^+$ T cells and HIV-specific $CD4^+$ and $CD8^+$ T cells [91]. Thus, other factors are of significance in stimulating the immune system. Bystander activation of various virus-specific cells contributes to expansion of activated $CD8^+$ T cells [91]. As mentioned in section 1.5.2, HIV mediated breakdown of the gut barrier with subsequent microbial translocation of microbial products as for instance LPS, is an important source of inflammation [66, 67, 72]. Co-infections such as CMV [99, 100] and hepatitis C (HCV) [101-103] are associated with increased immune activation and inflammation and lifestyle factors (e.g. smoking, excessive alcohol intake and obesity) may also play a role [76]. Dysfunctional immunoregulatory mechanisms with increased fractions of regulatory $CD4^+$ T cells (Tregs) [104], higher indoleamine 2,3 dioxygenase (IDO) activity [66] and disturbed Th17/Tregs balance [66] also seem to contributes [75, 77].

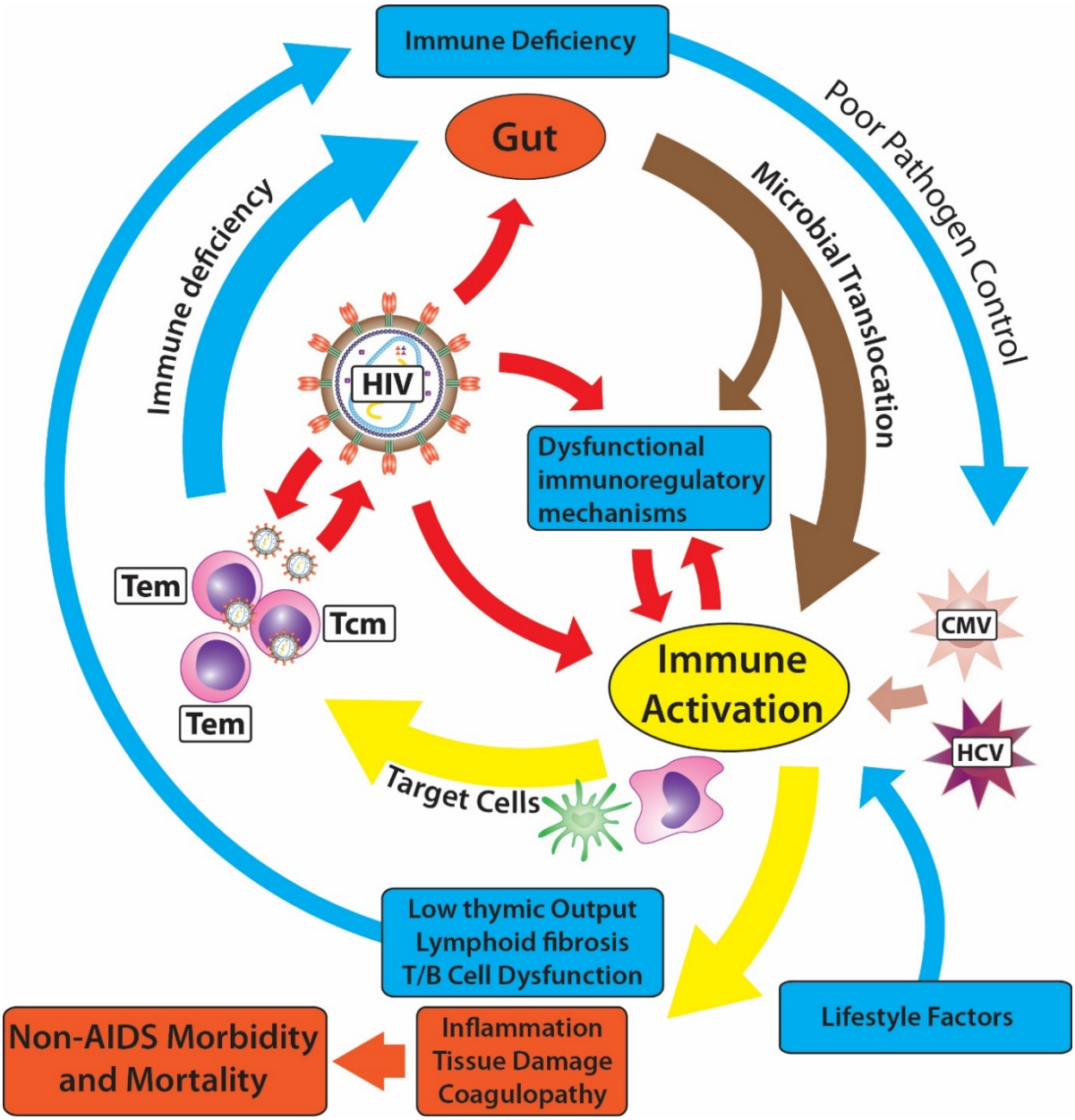


Figure 7. Possible contributors to a vicious circle of chronic immune activation in HIV infection. HIV replication and immune response to virus drives the immune activation in untreated patients. HIV-induced damage to the gut mucosal barrier and immunity results in microbial translocation and cause local and systemic immune activation and inflammation. Induction of dysfunctional immunoregulatory mechanisms can potentially aggravate immune activation. The subsequent expansion of EM $CD4^+$ T cells increases the number of target cells for HIV and escalates HIV replication. Direct and indirect mechanisms cause $CD4^+$ T cell loss. Chronic activation of the immune system results in reduced thymopoiesis and damage to lymphoid tissues, contributing to impaired T cell regeneration and overall decreases function of adaptive and innate immunity. The resulting immunodeficiency leads to co-infections, boosting immune activation. Also, lifestyle factors can influence chronic immune activation. This chronic inflammatory state contributes to the development of AIDS and non-AIDS morbidity.

1.5.4 T cell activation, differentiation and exhaustion

$CD8^+$ T cell activation characterized by expression of CD38 or co-expression of HLA-DR is an important predictor for disease progression independent of the level of viremia and CD4 count in untreated HIV infection [79, 81, 82]. High $CD8^+$ and $CD4^+$ T cell activation pre-ART are associated with reduced CD4 gain on treatment [105, 106]. During ART, T cell activation is reduced and weakened as a predictor for clinical outcome [81, 83, 106-108]. Studies of treated PLWH have not found any link between $CD8^+$ or $CD4^+$ T cell activation and serious non-AIDS events (SNAE) [83, 109] when adjusting for several factors including the CD4 count. An exception is a Ugandan cohort where HLA-DR and CD38 co-expression predicted mortality also in virally suppressed patients [106], indicating that adaptive immune defects could be of higher importance in PLWH in resource limited settings. Persistent $CD8^+$ T cell activation in ART treated seems to be associated with lower nadir CD4, low-level viremia, reduced $CD4^+$ gain [107, 110] and frailty [110].

The T cell activation drives the turnover and differentiation of the T cells from naïve to highly differentiated antigen-experienced cells [52, 89, 90, 111, 112]. Activation of naïve T cells may lead to depletion of these cells by apoptosis or proliferation into memory cells [111], whereas higher age and the HIV infection attenuate the generation of naïve cells from the bone marrow and thymus [113-115]. Progressive destruction of lymph node architecture contributes to the impaired T cell homeostasis and loss of naïve T cells by increased apoptosis of these cells [52, 116]. In sum, the result is skewing of the T cell population towards reduced numbers and fractions of naïve $CD4^+$ and $CD8^+$ T cells and increased frequencies of EM $CD4^+$ and $CD8^+$ T cells.

After initiation of ART, T cell activation and the rate of T cell turnover decreases [88, 112, 117-119]. The regeneration of naïve T cells takes longer time and the capacity is limited, especially in older age and with more advanced disease pre-ART [85], leading to the fact that higher fractions of memory T cells are often seen, also on ART.

Persistent exposure to antigen and chronic immune activation causes exhausted HIV-specific T cells characterized by loss of effector functions and increased expression

of inhibitory surface molecules. Programmed death 1 (PD-1) is well-known as a marker of T cell exhaustion and is highly expressed on HIV-specific CTLs [120-122]. It inhibits HIV-specific T cell activation and proliferation by presenting negative costimulatory signals to T cells and is involved in apoptosis [120, 122]. PD-1 expression on T cells correlates with disease progression and is reduced during ART [120, 122]. Blockade of PD-1 *in vitro* has shown to improve HIV-specific CTL function and T cell proliferation [120, 122] and *in vivo* in SIV-infected, increased virus-specific $CD8^+$ T cell responses, reduced viral load and prolonged survival [123].

1.5.5 $CD4^+$ T cell decline

Another hallmark of HIV infection is the progressive loss of $CD4^+$ T cells caused by increased destruction and reduced production. Together with $CD8^+$ T cell activation, the CD4 counts are the best characterized biomarkers of disease stage in untreated HIV infection [75]. Several factors contribute to the depletion of $CD4^+$ T cells. Overall, HIV destroys mature effector $CD4^+$ T cells directly or indirectly by virus-specific CTL and at the same time impairs the regeneration of new progenitor cells [115, 124]. However, during chronic infection the frequency of infected $CD4^+$ T cells is too low to account solely for this decline [87, 125], and the extent of the $CD4^+$ T cell depletion correlates more closely with levels of immune activation than the viral load [126]. Activation-induced apoptosis related to the chronic high levels of generalized immune activation is considered as a major cause of peripheral $CD4^+$ T cell loss in PLWH [111, 127]. In addition, abortive infection with incomplete reverse transcription in non-permissive resting $CD4^+$ T cells in lymphoid tissue, probably results in pyroptosis related death of a substantial number of local $CD4^+$ T cells [55]. Pyroptosis causes release of several inflammatory mediators contributing to pyroptosis of neighboring cells, more chronic inflammation as well as attraction of new cells to die [55, 128].

Coincident with the $CD4^+$ T cell depletion, HIV infection also affects the capacity of the immune system to regenerate new $CD4^+$ T cells [115]. Lymphopoiesis is suppressed, probably due to the systemic immune activation [114] and a decrease in thymic volume and function is observed [113, 129]. The extent of collagen deposition in lymph nodes

seems to increase with disease progression and have negative impact on both apoptosis of naïve T cells (increased) and the capacity to regenerate these cells [116, 130].

The loss of $CD4^+$ T cells and in particular the naïve population results in reduced T cell repertoire and defense against other micro-organisms. When the $CD4^+$ T cell population declines, increased general infection rates contributes to higher immune activation and accelerated loss of $CD4^+$ T cells. In late stages of HIV-disease, viruses using the CXCR4 co-receptor become dominant, thereby increasing the infectiousness further since this receptor is expressed by both naïve and resting central memory (CM) cells [90].

1.5.6 Regulatory T cells (Tregs)

Tregs can suppress the activation, proliferation and effector functions of a wide range of immune cells [131]. Their presence is crucial to prevent development of autoimmune diseases and allergy, to maintain tolerance during pregnancy and after organ transplantation, and to downregulate immune reactions to foreign antigens [131]. On the other side, Tregs can also suppress beneficial antitumor and HIV-specific responses [131, 132]. Tregs induce suppression through several mechanisms; as for instance expression of cytotoxic T lymphocyte Antigen (CTLA)-4 that prevents T cell activation by binding to CD80 and CD86 on antigen-presenting cells (APCs), expression of CD39 and CD73, which affects adenosine signaling, consumption of IL-2 and secretion of the suppressive cytokines IL-10 and TGF-β [131].

Tregs accumulate in lymphoid and mucosal tissue in chronic HIV infection [133-135]. Still, regardless of the Tregs phenotype analyzed, several studies show increased fractions of Tregs in chronic HIV infection [104, 134-137], being highest among those with reduced CD4 count [134, 136-138], whereas the relation to viremia is more unclear [132]. During ART, the frequencies of Tregs seem to decrease and even normalize [134, 136, 139, 140] and interestingly, HIV controllers are reported to have low fraction of Tregs [104, 132, 134].

Data indicate that Tregs suppress HIV-specific T cell activity in all stages of HIV disease, as Tregs from both acute, chronic infected and virally suppressed PLWH have been shown to suppress HIV-specific and other stimuli-induced proliferative $CD4^+$ T

cell responses [104, 133, 141, 142]. Moreover, Tregs depletion *in vitro* has increased $CD4^+$ and $CD8^+$ T cell responses to both HIV, other viruses and TCR stimulation [133, 141-144]. In HIV controllers protective HIV-specific $CD8^+$ T cells seem to evade suppression by Tregs [145]. Furthermore, in untreated the fractions of Tregs have mainly been positively associated with chronic immune activation [104, 132]. On the other side, Tregs might partly reduce harmful general immune activation during HIV infection [132]. This demonstrates the opposing roles of Tregs' presence in HIV infection; suppression of HIV- and other pathogen-specific beneficial responses and concomitantly partially dampening the chronic immune activation.

Tregs can be divided into naïve/resting Tregs (rTregs) that are long-lived, have suppressive capacity and are able to differentiate into activated Tregs (aTregs), which yield strong suppression and then die by apoptosis [131]. Compared to healthy controls, HIV-infected with and without ART may have reduced fractions of rTregs partly due to lower thymic output and excessive conversion to aTregs [136, 146], accompanied by higher percentages of aTregs [146]. Studies diverge in whether the Tregs subsets are normalized or not on ART [136, 139, 146-148]. Reporting Tregs subsets as percentages of $CD4^+$ T cells or Tregs and various definitions of the subsets can contribute to these different results.

Targeted manipulation of Tregs responses could potentially play a role during HIV infection, but remains to be clarified [149].

1.5.7 Disruption of the gut barrier and microbial translocation

A healthy gut barrier consists of a continuous layer of enterocytes with intact tight junctions, a high luminal Immunoglobulin (Ig) A concentration and various functional immune cells such as DCs, macrophages, T cells and innate lymphoid cells in the lamina propria [73, 74]. Together with the preferentially loss of the IL-17 producing gut mucosal cells (see section 1.5.2), other immunological alterations and breaches in the mucosal barrier also characterize mucosal dysfunction in PLWH. Depletion of Th22 cells secreting IL-22 [150-152], presence of increased proportion of IDO expressing DCs [66], loss of IL-17 and IL-22 producing natural killer (NK) cells and dysregulation

of γδ cells probably play a role [73, 153]. The integrity of the epithelial barrier shows signs of disruption with loss of tight junctions, enterocyte apoptosis and increased intestinal permeability [64, 69, 73, 74, 154]. This allows for persistent translocation of not only LPS, but also other microbial products from bacteria and fungi such as flagellin, peptidoglycan, lipoteichoic acid, ribosomal DNA and unmethylated CpG-containing DNA into lamina propria and the systemic circulation contributing to chronic immune activation [67, 71, 72, 155]. The microbial products binds to pattern recognition receptors (PRR) on innate immune cells causing local inflammation. Like a vicious circle this can aggravate local tissue damage and cause further disruption of the gut immune defense and barrier. Despite suppression of HIV replication by ART, restoration of gut mucosal barrier and gut mucosal immunity occurs slower and is often incomplete [57, 153, 156, 157]. Only initiation of ART in the very early stages have been shown to restore the proportion and function of Th17 cells [65]. Consistent with this partial restoration of gut immunity, levels of microbial translocation are reduced on ART, but remain generally elevated compared to healthy individuals [67, 71, 72, 158].

Plasma LPS has been extensively analyzed in PLWH on ART and remains elevated, but have together with other markers of microbial products not yet been linked to increased mortality [67, 72, 158]. Nevertheless, LPS seems to be associated with subclinical atherosclerosis [159], inflammatory intermediate monocytes [160] and increased monocyte tissue factor (TF) expression, which initiates the coagulation cascade on the surface of monocytes [161].

On the other side, markers of gut barrier disruption analyzed in plasma such as intestinal fatty acid binding protein (I-FABP) released upon gut epithelial cell death, and zonulin-1 reflecting tight junction dysfunction, have been shown to predict mortality in treated HIV infection, mainly in those with advanced disease prior ART start [109]. Detectable I-FABP levels have also been associated with lower nadir and baseline CD4 counts supporting the assumption that gut mucosal damage contribute to SNAE to a greater extent in patients with marked immunodeficiency pre-ART [76, 158].

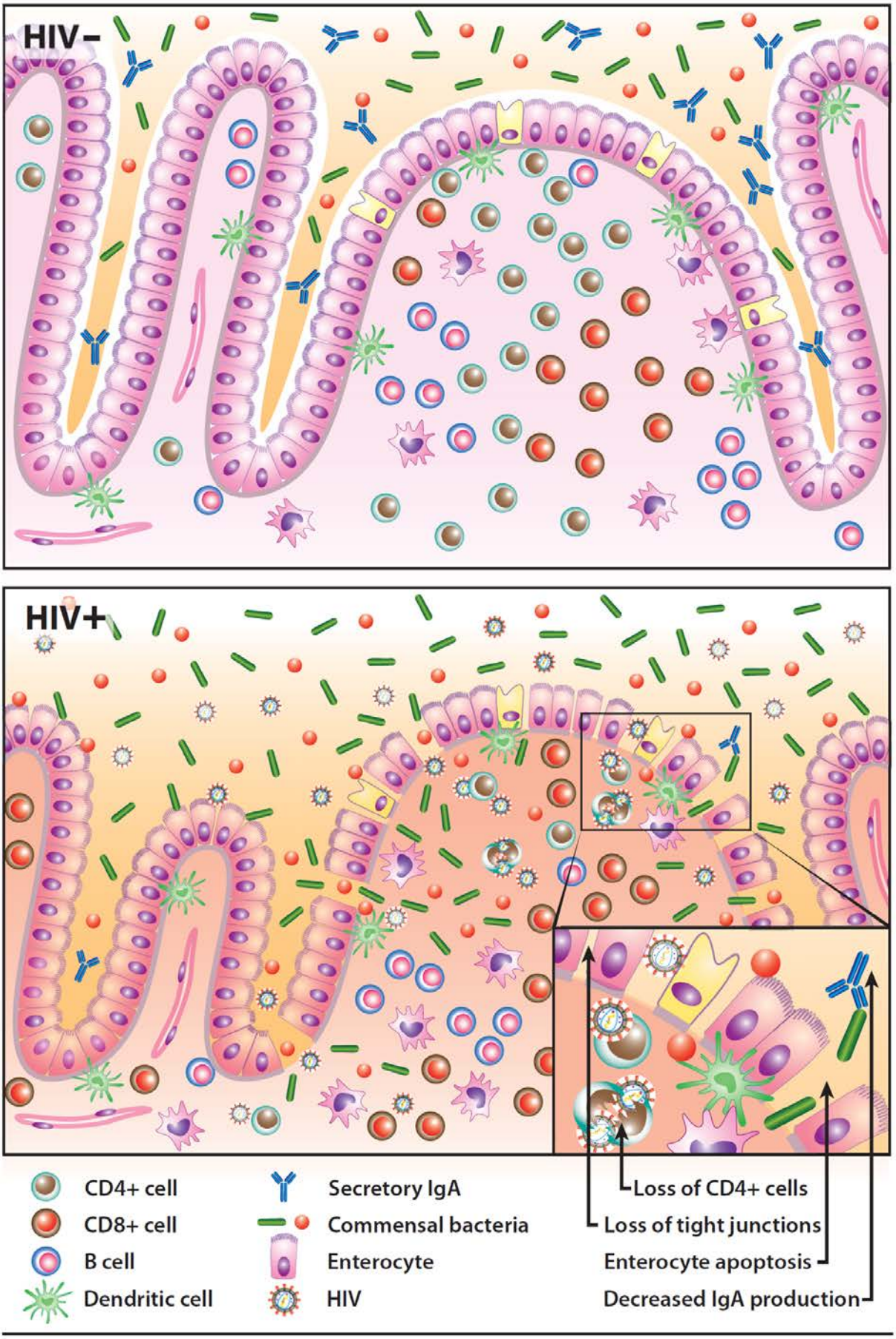

HIV–
HIV+
CD4+ cell
CD8+ cell
B cell
Dendritic cell
Secretory IgA
Commensal bacteria
Enterocyte
HIV
Loss of CD4+ cells
Loss of tight junctions
Enterocyte apoptosis
Decreased IgA production

Figure 8. Impact of HIV on gut mucosal immunity and barrier. The healthy gut mucosa (top panel) has functional tight epithelial junctions and well-functioning DCs, macrophages, neutrophils, $CD4^+$, $CD8^+$ T cells and B cells. Also, a layer of protective mucus, antimicrobial peptides and secreted IgA contribute to limit systemic exposure to commensal bacteria and microbial products. HIV infection alters the gut mucosal immunity and barrier (lower panel), causing breakdown in tight junctions, loss of resident immune cells, Th17 cells in particular, immune dysregulation, reduced IgA secretion, changes in gut microbiota, leading to microbial translocation and chronic inflammation.

1.5.8 HIV-related dysbiosis

The human GI tract is colonized with approximately 10^{14} microorganisms and tend to increase distally along the intestine. Their interactions with the host are important for gut homeostasis, immune processes and health [162, 163]. The gut bacterial genome consist mainly of the four phyla; the most dominant Firmicutes followed by Bacteriodetes, Actinobacteria and Proteobacteria [163] and the composition is influenced mainly by environmental factors such as diet and lifestyle, and to a lesser degree by genes [164]. In adults, the core microbiota is relatively stable within individuals, but changes can be induced by diet, infections and use of antibiotics [163]. Furthermore, alterations in the gut microbiome have been well documented in obesity and several diseases as for instance inflammatory bowel disease, atherosclerosis and type II diabetes [163].

Recently, it has become clear that PLWH have changes in the gut microbiota composition characterized by decreased abundances of bacteria important for gut homeostasis, in combination with increased abundance of bacteria with proinflammatory potential [165]. This so-called dysbiosis is associated with activation of immune cells in the gut lamina propria [151, 166, 167], microbial translocation and systemic immune activation and inflammation [166-172]. However, results are varying depending of the patient cohorts investigated, the sampling methods and the analyzing platforms used [165, 173]. Generally, dysbiosis is still present in most PLWH on ART [167, 169, 170, 174, 175].

Of note, sexual preference is a major shaper of the gut microbiome, and several findings in the early studies of the HIV-related gut microbiome was driven by MSM status rather than HIV status per se [176]. A shift in the Bactereoidetes phylum with increased relative abundance of the genus *Prevotella* with pro-inflammatory potential and a concomitant decreased abundance of the genus *Bacteroides* have been frequently reported changes in PLWH [166-168, 170, 174, 177]. Yet, in 2016 Noguera-Julian *et al* demonstrated that a high *Prevotella* / low *Bacteroides* fecal enterotype was associated with MSM sexual preferences independently of HIV status [176]. They found no evidence of a HIV-specific dysbiosis after stratifying for sexual orientation, suggesting that sexual behavior could have confounded conclusions from previous HIV microbiota studies.

Nevertheless, several studies describe reduced microbial diversity of the gut flora [168, 169, 175, 178] even when stratifying for sexual preferences (MSM vs non-MSM) [176]. In concordance with these results, Guillen *et al* demonstrated low microbial gene richness in PLWH regardless of sexual behavior [172]. Yet, the results are diverging as others have reported increased [174], or no differences in diversity in PLWH compared with healthy controls [166, 167, 170, 171].

With regards to alterations in the gut microbial composition following HIV infection, the overall findings have been enrichment of the phylum Proteobacteria and several subtaxa containing pathogenic bacteria, combined with a depletion of the phylum Firmicutes [166-168, 170, 175]. Importantly, only one of these studies was matched on risk groups [167]. Firmicutes consists of various bacteria with immune regulatory properties as for instance *Lactobacilli.* Lower levels of *Lactobacilli* are described in viremic PLWH compared with the general population [179], and higher levels of *Lacobacillales* have been associated with higher fractions or counts of $CD4^+$ T cells and reduced microbial translocation and systemic immune activation both in recently HIV-infected and during ART [171, 180]. Moreover, Firmicutes also contains species that produce the short-chain fatty acid butyrate, which is an important energy source for epithelial cells and also influences the colonic homeostasis in a positive direction [181].

Several of these butyrate-producing bacteria seem to be depleted in PLWH [166, 168, 175, 177].

Thus, the literature point towards an association between dysbiosis, HIV pathogenesis, inflammation and disease progression. However, there are several potential confounding factors and no causality has yet been demonstrated.

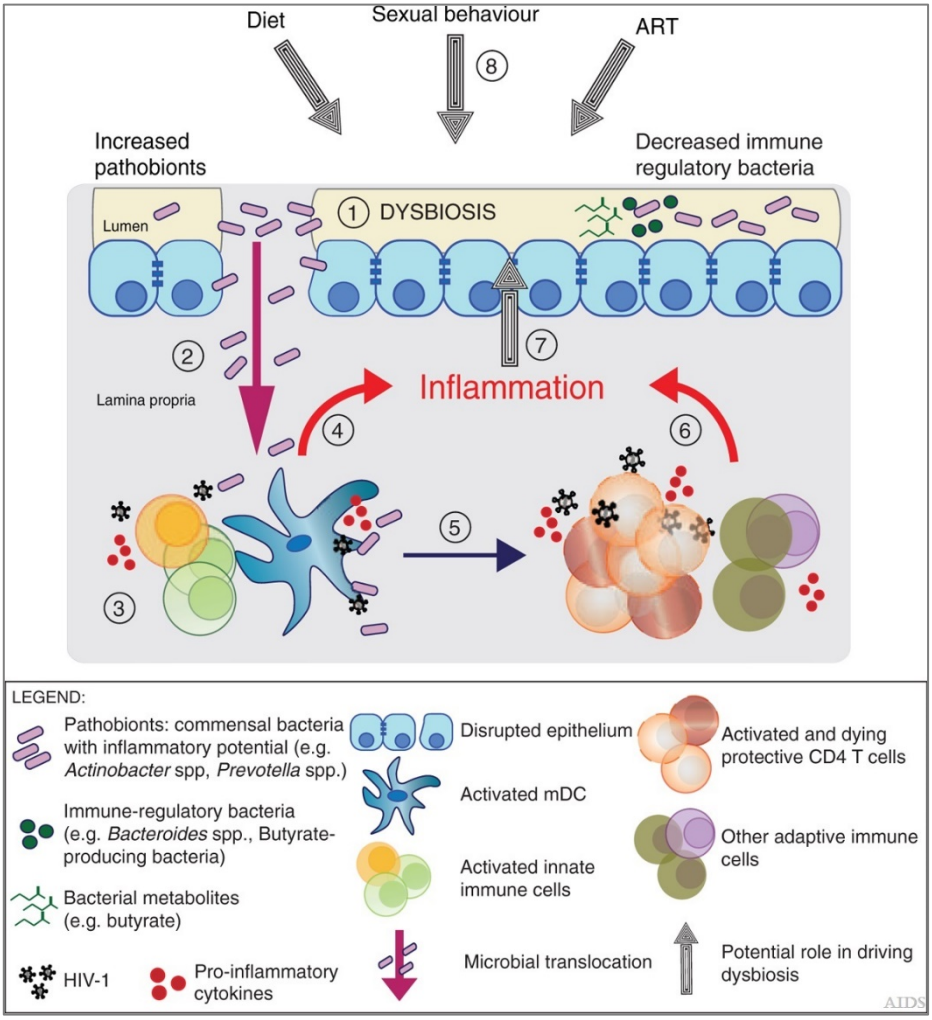


Figure 9. The gut microbiome and HIV pathogenesis. Presence of dysbiosis (1) and translocation of pathobionts through the disrupted epithelial barrier (2) cause activation of innate immune cells (3) and subsequently local inflammation, immune dysregulation (4) and increased $CD4^+$ T cell activation and death (5). Increased T cell activation aggravates the inflammatory environment (6) and together with the loss of important immune cells amplify epithelial barrier breakdown and may also promote dysbiosis (7). Lifestyle factors such as diet and sexual behavior can also contribute to alterations in the gut microbiome (8).

1.5.9 Indoleamine 2,3-dioxygenase (IDO) activity

IDO is an intracellular enzyme that converts the essential amino acid tryptophan to kynurenine [182]. It is part of the innate host defense and is predominantly found in monocytes, macrophages and DCs, but can be induced in a wide range of cells in response to immune activation and inflammation [182]. IDO regulates adaptive immune responses in a variety of diseases primarily by suppressing T cell responses [182].

IDO activity seems to be an important link between innate and adaptive immunity in HIV infection and kynurenine/tryptophan ratio (KTR) is used as a measure of IDO activity [183]. In 1998, Huengsberg *et al* showed that KTR was increased in HIV infection and associated with CD4 count and disease progression [184]. Since then, several studies have confirmed these results [66, 157] and that IDO activity is reduced on ART, but not normalized [109, 183, 185, 186]. Importantly, KTR independently predicts CD4 recovery [183], mortality [109, 183, 187], SNAE [83] and carotid artery atherosclerosis [188] in ART-treated PLWH.

The IDO activity is probably driven by HIV directly [189, 190] as well as by interferon (IFN)γ and microbial products such as LPS [66]. Both depletion of tryptophan and accumulation of toxic metabolites are thought to contribute to pathogenicity following increased tryptophan catabolism. Tryptophan depletion suppresses T cell responses [182] and local IDO activity with subsequent increase of quinolonic acid in the brain is associated with HIV-dementia [191]. Furthermore, tryptophan and its metabolites play an important role in gut mucosa immune tolerance. The loss of the mucosal Th17 population combined with generation of Tregs have been associated with IDO activity [66]. *In vitro,* this harmful effect on Th17/Treg balance was mediated by a downstream tryptophan catabolite (3-hydroxyanthranilic acid) [66]. HIV-infected plasmacytoid (p)DCs *in vitro* can also induce differentiation of naïve T cells into Tregs in an IDO-dependent manner [190]. Finally, dysbiosis of the mucosal adherent microbes leading to enrichment of bacteria with capacity to catabolize tryptophan via IDO seems to be related to higher KTR even in ART-treated [167], whereas *Lacobacillus* species probably can inhibit IDO activity [192]. Altogether, chronic IDO activity creates a vicious circle leading to increased Tregs combined with suppression of T cell responses

and depletion of Th17 cells, maintaining disruption of the gut barrier and microbial translocation, which further activates the IDO pathway and contribute to chronic immune activation (see figure 10).

Recent literature have also pointed KTR as a biomarker for active tuberculosis (TBC) in PLWH [193] and found that the IDO activity was independently associated with the size of HIV-reservoir [185].

Disappointingly, IDO blockade to ART-treated SIV-infected macaques showed only minimal effects on the tryptophan catabolism and did not decrease T cell- or innate immune activation and showed diverging impact on viral RNA [194, 195].

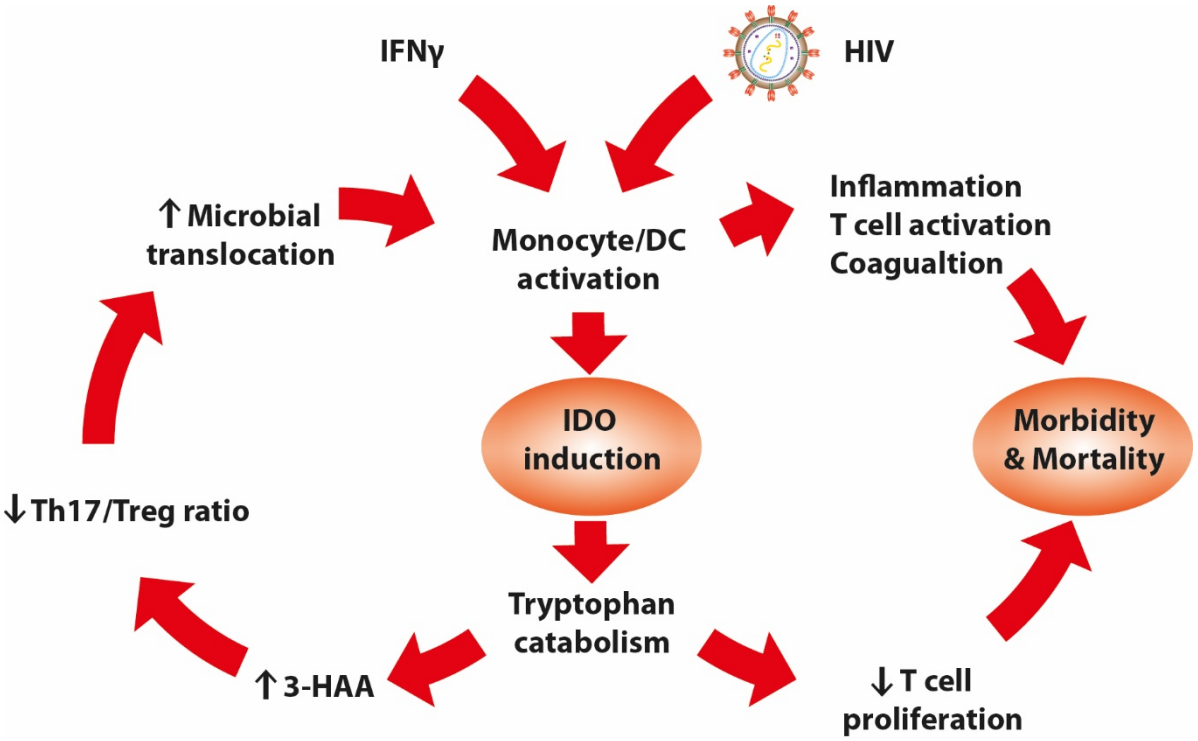


Figure 10. IDO pathway and HIV pathogenesis – a vicious circle. 3-HAA (3-hydroxyanthranilic acid)

Modified by permission from Prof. Peter William Hunt (personal correspondence 27.05.19)

1.5.10 Dendritic cells

DCs serve as a bridge between innate and adaptive immunity. Blood DCs are divided in two main subsets; pDCs and mDCs, which can be further subdivided into $CD141^+$ mDC and $CD1c^+$ mDC. mDCs are professional APCs and they recognize diverse pathogens due to their broad TLR expressions and C-type lectins [196, 197]. After antigen capture and processing in peripheral tissue, they mature and up-regulate co-stimulatory molecules, migrate to lymphoid organs for antigen-presentation to naïve T cells and induce T helper responses [198]. IL-12 production generates Th1 differentiation enhancing CTL- and phagocyte-mediated killing of intracellular microbes. pDCs express TLR7 and 9, are the major producers of IFNα, leading to stimulation of a wide range of immune cells in both the innate and adaptive immune system [199].

DCs play essential roles in HIV infection and pathogenesis, from recruitment of target cells, viral transmission and antiviral defense in the acute phase to chronic immune activation, T cell dysregulation and exhaustion in advanced disease. Understanding how DCs are affected during HIV infection is also important for vaccine strategies aiming to enhance cell-mediated immunity against HIV [196, 197]. DCs express high amounts of HIV-entry receptors CCR5 and CXCR4, less CD4, but also C-type lectin receptors such as DC-SIGN that bind HIV at its gp120 domain [197, 200]. Compared to T cells, very few DCs get productively infected with HIV [201, 202]. By binding of HIV to DC-SIGN, DCs can transfer HIV to uninfected T cells and promote viral dissemination [200, 202]. HIV activates pDCs directly, mainly through TLR7 [197, 203-205], whereas mDCs are reported to be dependent of exposure to pDCs derived cytokines to mature after exposure to HIV [203, 204].

Circulating mDCs and pDCs are reduced during acute HIV infection [157, 203, 206, 207]. Trafficking to lymphoid tissue, increased death and probably also reduced differentiation from bone marrow precursors contribute to the observed loss of DCs in the blood [208]. In untreated HIV infection the number of mDCs and pDCs seems to reflect the disease progression with an inverse correlation with the viral load and a positive correlation with the CD4 count [203, 207, 209-211]. However, there have been diverging results regarding expression of DC maturation markers and function as

measured by IFNα production in pDCs, or IL-12 secretion from mDCs after exogenous stimuli [203, 211-215]. Impaired ability of both mDCs and pDCs to stimulate T cell proliferation *in vitro* has been observed [216]. On the contrary, elite controllers are reported to have increased antigen-presenting function of mDCs combined with reduced pro-inflammatory cytokine production after TLR stimuli [217].

Unlike other viruses, HIV can apparently drive a continuous IFNα secretion [218] and together with other immunomodulatory factors such as products of microbial translocation [67, 151], contribute to the persistent IFNα production often seen in chronic HIV infection [202]. Prolonged IFNα signaling in the end promotes T cell dysregulation, exhaustion and apoptosis [202, 219, 220]. Sooty mangabeys, the natural hosts of SIV, are shown to have reduced type I interferon gene expressions during chronic SIV-infection compared to SIV and HIV-infected rhesus macaques and humans [221]. In line with this observation, human viremic non-progressors displayed lower levels of interferon-stimulated genes than progressors [222]. Finally, in a SIV model, type I interferons were important for antiviral control during acute infection and future prognosis, whereas continuous IFNα2 treatment was associated with disease progression [223].

Furthermore, upregulation of IDO in DCs [66, 189, 190] promotes differentiation of Tregs that could contribute to HIV pathogenesis (see section 1.5.9). Finally, during progressive HIV infection the crosstalk between DCs and NK cells has been reported to be disrupted [197].

In virally suppressed PLWH most studies show a normalization of the mDC counts and fraction [157, 211, 224], also in immunological non-responders (INR) [225], while the restoration of pDCs is more variable and probably takes longer time [157, 203, 207, 211, 225, 226]. Despite viral suppression there are still signs of *in vitro* DC dysregulation as some demonstrate weakened pDC IFNα secretion, but preserved or increased production of pro-inflammatory cytokines after exogenous stimuli [225-227], and impaired induction of Th1 responses by monocyte-derived DCs [228]. In contrast, others have found that IFNα production after TLR challenge returns to normal level on ART [213].

Recently, pDC activation or IFNα intervention has been suggested as potential agents in the HIV cure strategy [229]. Two pilot trials address this question in ART-treated P. First, IFNα2 intervention was reported to reduce integrated HIV DNA and control HIV viremia after ART interruption [230]. TLR9 agonist treatment boosted NK and $CD8^+$ T cell activity via activation of pDCs, enhanced HIV-specific $CD8^+$ T cells, but did not reduce HIV DNA or improve viral control after ART interruption [229, 231]. Just recently, Kristoff *et al* showed in *ex vivo* experiments that HIV-antigen stimulated monocyte-derived DCs generated from HIV-infected individuals on ART could both reverse HIV latency in infected autologous $CD4^+$ T cells and also activate HIV-specific $CD8^+$ T cells [232]. Whether these monocyte-derived DCs could be effective in the "kick and kill" strategy *in vivo*, remains to be seen.

Taken together, DCs are important for the acute antiviral defense and viral transmission, contribute to persistent immune activation during chronic HIV infection and even on ART, pathological changes in this cell subset probably remain. Modulation of pathogenic pathways initiated by DCs might be useful both in vaccination and HIV cure strategies. Finally, data on DCs in INR are sparse.

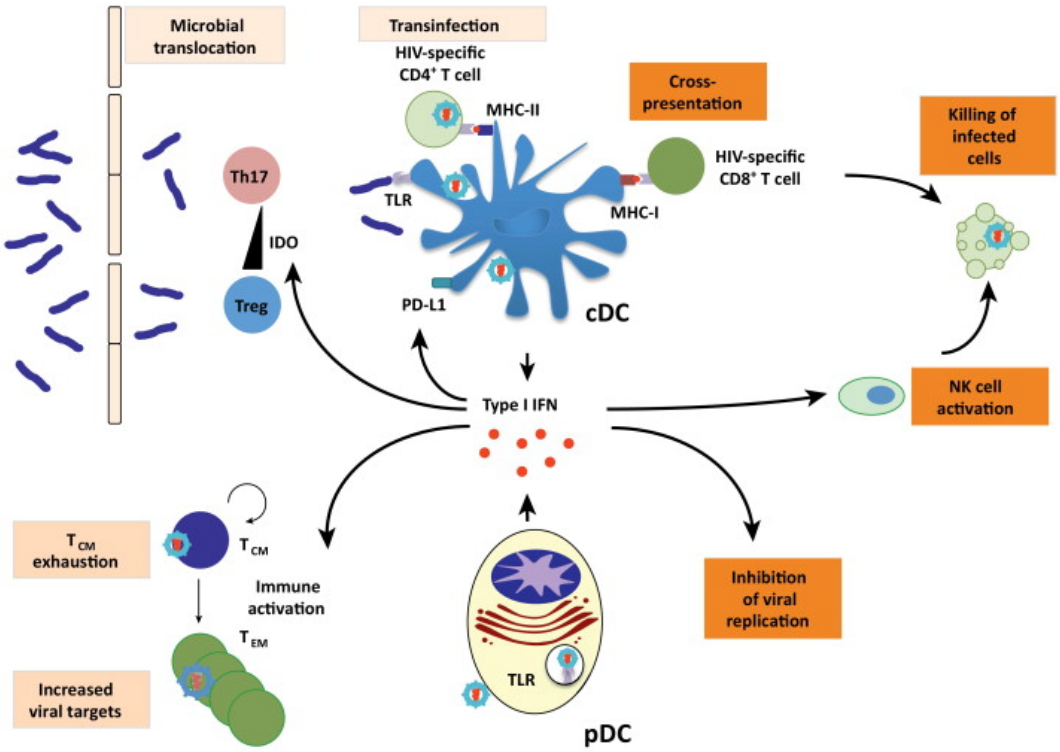


Figure 11. Conflicting role of DCs in HIV infection. Conventional DCs (cDCs =mDCs) and pDCs both contribute to elimination of HIV-infected cells. Activation of mDCs leads to CTL- and phagocyte-mediated killing of HIV-infected cells. DCs and NK cells crosstalk, causing activation of NK cells and elimination of HIV-infected cells and type I IFN can inhibit HIV replication. In contrast, DCs also contribute to the pathological immune activation observed in chronic HIV infection. Type I IFN and increased IDO expression lead to reduced Th17/Treg ratio in the gut, disturbing the gut immunity and promoting microbial translocation. Persistent inflammatory cytokines and IFN secretion generate more target cells for HIV and can also cause exhaustion of T cells. In addition, transinfection can facilitate viral spread.

1.5.11 Monocytes

Monocytes are derived from hematological bone marrow precursors and enter the blood circulation. They can develop in several different ways, influence the adaptive immune system and posess important roles in trafficking and presentation of antigens to T cells [233]. Monocytes are differentiated into three main subsets based on their CD16 and CD14 expression; classical: $CD14^{++}CD16^{-}$, intermediate: $CD14^{++}CD16^{+}$ and non-classical: $CD14^{+}CD16^{++}$ monocytes [234]. Classical monocytes are rapidly recruited and very short-lived and give either rise to intermediate monocytes, or disappear by death or migration [235]. They encompass high antimicrobial and phagocytic capacity and the capability to transmigrate across the endothelium upon recruitment to sites of injury and infection [233, 236]. In tissues the may remain as monocytes, or mature into inflammatory macrophages or monocyte-derived DCs [233]. Intermediate monocytes are in transition from classical to non-classical monocytes, but still represent a unique cell population [237]. They are major producers of inflammatory cytokines, express high levels of MHC II, have high capacity for antigen presentation [236] and circulate for a few days [235]. Non-classical monocytes patrol the endothelium in blood vessels, have endothelial cell-supporting functions, produce proinflammatory cytokines and circulate for the longest period, for about a week [235, 238].

In HIV-uninfected people, monocytes play a major role in the initiation and progression of atherosclerosis [239]. Activation of monocytes also seems to be related to higher age

in both PLWH and uninfected people [240-242]. Increased activation of monocytes has been reported even in ART-treated PLWH and has been connected to cardiovascular disease and markers of inflammation and coagulation [160, 243-246]. More precisely, increased percentages of various activation markers [243, 247] and higher production of inflammatory cytokines [243, 246] are observed. In viremic PLWH, most studies have shown higher proportions of intermediate monocytes compared to healthy controls, whereas levels in PLWH on ART appear to be normalized [160, 240, 243]. Increased percentages have also been reported in elite controllers [248]. The frequencies of CD16 positive or intermediate monocytes and the monocyte cytokine production are correlated to both markers of coagulation and inflammation [160, 243, 244] and the CD16 fraction also to coronary artery calcium progression [245], but not with carotid intima thickness [249]. Moreover, several studies have demonstrated increased expression of TF in monocytes in PLWP, both untreated and on ART, compared with healthy controls [246, 248]. This upregulation is thought to be partly driven by circulating microbial products [161, 246]. Funderburg *et al* reported fractions of TF positive monocytes almost in level with uninfected patients with acute coronary syndrome [160]. In addition, TF expressing monocytes were associated with SIV-related coagulopathy in rhesus macaques, but not in African green monkeys with non-pathogen SIV [246].

Finally, circulating biomarkers released by activated monocytes, such as IL-6, interferon-inducible protein 10 (IP-10), neopterin and in particular soluble (s)CD14 and sCD163 are often used to characterize monocyte activation.

All together this suggests that HIV infection both directly and with contribution from for instance microbial products, may generate activated monocytes contributing to SNAE and in particular increased cardiovascular risk.

1.5.12 Soluble inflammation markers

Several soluble inflammatory markers increase early during the acute infection [48], remain elevated during the chronic phase [250, 251] and are associated with markers of disease progression such as T cell activation, CD4 counts and level of viremia [77, 252]. During ART the levels are generally reduced, but usually not normalized [83, 187, 250,

252-254]. In 2008 Kuller *et al* showed in the SMART study, that IL-6, D-dimer and high sensitivity C-reactive protein (hs-CRP) were strongly related to all-cause mortality, in particular in those with continuous ART [255]. Later on, numerous studies across different cohorts have demonstrated associations between levels of many different soluble inflammation markers and mortality and non-AIDS morbidity in treated HIV infection [75, 76, 83, 109, 187, 252, 256-258], even among those with high CD4 count [256-258], although the associations appeared to be more pronounced in those with most advanced immunodeficiency [109]. Results are consistent for most markers analyzed both years before and just prior SNAE [83, 255, 256, 258]. After one year on ART, the soluble biomarker levels are reported to be fairly stable [250, 252]. Importantly, increases thereafter seem to precede a SNAE [252]. Finally, chronic inflammation is probably a more important determinant of mortality and morbidity in PLWH than in the general population [76, 259].

IL-6 is released from various cells, in particular monocytes and macrophages [260]. Plasma IL-6 is one of the best documented biomarkers which is consistently and strongly related to SNAE, in particular mortality, in PLWH on ART [83, 109, 252, 255, 256, 258, 261]. More precisely, in addition to mortality, IL-6 is independently associated with cardiovascular disease [256, 261], non-AIDS related malignancies [256] and low functional impairment [110].

hsCRP is an acute phase reactant produced by the liver after stimulation from IL-6 in particular [260], and is in line with IL-6 associated with SNAE overall [109, 255, 256], cardiovascular events [261] and non-AIDS related malignancies [256], although not to the same extent as IL-6 [109, 256].

Coagulation and inflammation are closely related processes [262] and markers of inflammation correlate with **D-dimer** in treated HIV infection [71, 109, 161]. D-dimer is a fibrinogen degradation product and a marker of active coagulation [263]. Higher levels of D-dimer are independently related to SNAE overall [83, 252], mortality [109, 187, 255] and cardiovascular disease [261, 264].

sCD14 and sCD163 are generally considered as markers of monocyte activation (see section 1.5.11). CD14 is co-receptor for LPS on monocytes, but several other inflammatory stimuli can cause elevated levels of plasma sCD14 and sCD14 is probably a non-specific marker of monocyte activation [265]. sCD163 is shed from activated monocytes and tissue macrophages and mediates clearance of hemoglobin [266]. sCD14 is extensively documented [109, 158, 187, 258], but a recent study also linked sCD163 independently to mortality [257]. sCD14 is associated with SNAE overall [83, 252] and both markers have been inconsistently related to subclinical cardiovascular disease [159, 267-269].

Finally, in addition to these well-documented markers, several other inflammation markers such as soluble tumor necrosis factor receptor I and II and IL-2 receptor are also associated with mortality [109, 258] or non-AIDS morbidity in PLWH on ART [83, 252].

1.5.13 IP-10

IP-10 is a pro-inflammatory chemokine secreted from INFγ stimulated immune cells following a Th1 response or NK cell activation. It binds to the CXCR3 receptor on various immune cells and is involved primarily in chemotaxis of these cells to inflamed area, but mediates also apoptosis, cell growth inhibition and angiostasis [270]. Increased levels have been observed in different viral infections [270], disease progression in HCV [271] and linked to atherogenesis by attraction of T effector cells [272].

IP-10 increases early during acute HIV infection [48] and it is widely documented that IP-10 levels remain elevated throughout the infection [92, 114, 250, 251, 273-277]. In untreated PLWH, IP-10 is strongly correlated with the level of viremia [72, 277-279], $CD4^+$ and $CD8^+$ T cell activation [278, 280] and reported to be a predictor of a more rapid disease progression [279, 280]. In contrast, HIV controllers display levels of IP-10 comparable to those on ART [92, 279]. Furthermore, IP-10 values are reduced during ART although most studies show persistently higher levels compared with healthy controls [92, 250, 273, 274, 276, 277, 279]. Based on its association with HIV RNA levels, IP-10 is suggested as a screening tool both to identify acute HIV infection in

febrile patients [275] and for detectable viremia in ART-treated in resource-limited settings [277, 281]. PLWH with CMV co-infection have apparently higher IP-10 levels than ART-treated CMV seronegative [282]. Moreover, high IP-10 values are reported to discriminate between active TBC disease or not in PLWH [283, 284]. Presence of gut dysbiosis is also linked to increased plasma IP-10 [167]. Finally, in the study by Tenorio *et al* high IP-10 levels independently predicted a SNAE, however only when analyzed immediately preceding the events [83].

1.6 Clinical prognosis and immunological response to ART

1.6.1 Clinical prognosis in ART-treated PLWH

Despite improved HIV care and access to ART, clinical challenges remain. Significant gaps in life expectancies persist between PLWP and the general population, between regions, risk groups and genders [285, 286]. Life expectancy at age 20 for PLWH on ART in 2008-11 expressed as the percentages of the life expectancy in the HIV-negative or the general population, ranged from 60% in Rwanda to ca 89% in Canada [285]. For well-treated in high-income countries, the life-expectancy approaches the HIV-negative population [286-288], whereas the older population, intravenous drug abuse (IDU), not virally suppressed and those who started ART during advanced disease continue to have increased age-related morbidity and mortality [286-290].

SNAE represent the major cause of the increased morbidity and mortality during ART [291-293]. Even compared with a high-risk control group and after adjustment for relevant risk factors, hypertension [289], cardiovascular disease [289, 290] and impaired renal function [289] are reported to be more prevalent in PLWP on ART[289]. Various cancers are diagnosed at younger age [294] and higher low-energy fracture rates are seen [295], although lifestyle factors can contribute to these higher incidences.

Confounding lifestyle factors such as smoking, alcohol abuse, illicit drug use, obesity etc. can explain some of the increased age-related morbidity and mortality [296-298]. However, persistent immune activation and inflammation predict these events [109,

158, 255, 289] and appear to play a major role. Direct toxic effects of ART, in particular older regimens, may also contribute [291].

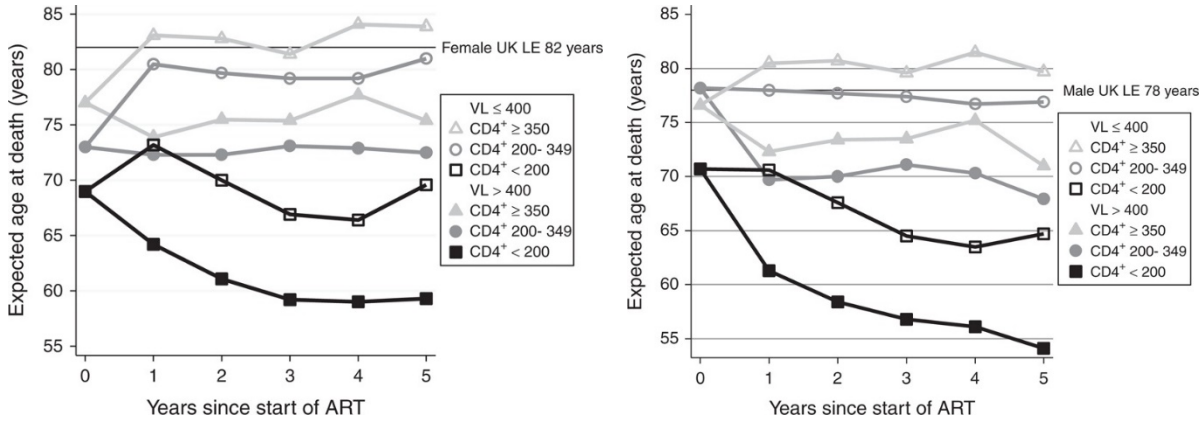


Figure 12. Life expectancy. Expected age at death of women (right panel) and men (left panel) aged 35 years at different durations of antiretroviral therapy according to current CD4 cell count and viral suppression compared with the general population.

1.6.2 Normal CD4$^+$ T cell response to ART

The initial peripheral CD4 response to ART is a consequence of rapid redistribution of mainly memory CD4$^+$ T cells from lymphoid tissues rather than de novo synthesis [88, 299, 300]. Previous studies have reported increases of CD4 count of 50-120 cells/µL in peripheral blood during the first three months [301-303], however earlier ART start seems to give a higher initial CD4 gain [304]. This phase is followed by a more gradually raise in CD4$^+$ T cells at an average rate of 2-7 CD4 cells/µL per month [301-303, 305, 306]. There is a larger contribution of naïve cells [299], which can be generated from thymopoiesis or homeostatic proliferation in the periphery. After 3-4 years the rate declines to around 1.5-2 CD4 cells/µL per month [301, 305-307] and for some individuals the CD4 count reaches a plateau [305, 307].

Most studies define an increase in CD4 count to >500 cells/µL as adequate [305, 307, 308] as patients with this level of immune restoration have life expectancy and morbidity

approaching HIV-negative persons [309]. However, current research has found that also CD4 counts above this level are associated with stepwise reduced risk of developing AIDS [310, 311] and a considerable fraction of non-AIDS related events occur indeed in ART-treated with CD4 count >500 cells/µL [40].

1.6.3 Immunological non-response to ART

Despite modern ART and persistent viral suppression, a substantial proportion of PLWH do not normalize their CD4 count [305, 307, 308, 312]. It is widely documented that the probability of achieving a normal CD4 count on ART is dependent of the pre-ART CD4 count [301, 304, 305, 307, 308, 310, 312-315]. Both previous studies and recent data from PLWH initiating ART after 2004 have shown that even after long-term ART with viral suppression for 6-7 years, 50-70% of patients with pre-ART CD4 counts <100 cells/µL and 25% with pre-ART CD4 counts 100-200 cells/µL did not obtain a CD4 count >500 cells/µL [305, 307, 308, 312]. In comparison, 85-95% of those with pre-ART CD4 >300-350 cells/µL reached this goal [305, 307, 308, 312].

Yet, there is no consensus regarding the definition of immunologic non-response to ART. Both failure to achieve a pre-specified raise in the CD4 count (50-100 cells/µL in 6-12 months) or to reach an absolute CD4 value (<200-500 cells/µL) [316-319] within a period are used as criteria. The most restrictive categorization of INR as persistent CD4 count < 200 cells/µL even after several years with ART has robustly been described as a group of patients with poorer prognosis [298, 314, 315]. Yet, CD4 gain to <350-500 cells/µL is also often used to determine immunological failure [315, 320-325]. Consequently, the percentages of INR varies between 12-30% depending of the definition and the population studied [298, 305, 307, 308, 312, 315]. Given the substantial proportion of PLWH who still enter HIV care and ART as late presenters in both high-and low- income countries, INR will continue to be of clinical relevance [326, 327].

1.6.3.1 Risk factors and mechanisms in INR

Low nadir and / or pre-ART CD4 counts are the strongest predictors for an incomplete immune reconstitution on ART. Long duration of HIV infection prior to ART potentially limits the restauration of the immune system and $CD4^+$ T cells [301]. Furthermore, older age is also highly associated with a lower CD4 regenerative response during treatment [298, 302, 305, 308, 312, 314, 315, 325, 328, 329]. However, the underlying mechanisms are complex and incompletely understood, and several factors contributing to reduced production as well as increased destruction and turnover, seem to play a role [316-318, 330].

Hematopoiesis is commonly affected in untreated HIV infection [115] and persistent damage of the lymphoid system or impaired lymphopoiesis are linked to reduced $CD4^+$ T cell recovery on ART [316, 317]. In INR with low nadir CD4 counts, bone marrow progenitor cells are reported to have decreased clonogenic potential [331]. Moreover, lower numbers of circulating $CD34^+$ hematopoietic progenitor cells have been associated with lower CD4 counts and reduced fractions of naïve $CD4^+$ T cells after several years on ART [114]. The T cell receptor rearrangement and generation take place in the thymus and a functional thymopoiesis is essential for reconstitution of $CD4^+$ T cells with a broad immunologic repertoire and could be compromised in INR [316, 317]. Small thymic size is associated with low CD4 gain [332] and reduced fractions of recent thymic immigrants, and in particular naïve $CD4^+$ T cells, are seen in INR [325, 333-336]. The fact that thymic tissue and function decreases with age could contribute to the observed robust linkage between older age and reduced $CD4^+$ T cell recovery. Furthermore, IL-7 which is crucial for proliferation of T cells in the thymus and survival of naïve and memory T cells, are found in higher concentrations in INR, but they are seem to have lower expression of the IL-7 receptor [331, 334, 335, 337-339]. Data from small studies indicate that the capacity of $CD4^+$ T cells to respond to IL-7 can be reduced [337-339] and genetic variations in the IL-7 receptor as well as limited access to IL-7 caused by destructed lymphoid architecture, could possibly affect the $CD4^+$ T cell response [116, 340].

In lymphoid tissue irreversible changes with collagen deposition and fibrosis can prevent normal $CD4^+$ T cell survival, proliferation and trafficking [341]. The extent of these changes in GALT and lymph nodes have been associated with a subsequent lower $CD4^+$ T cell recovery on ART [116, 130, 341-343].

Altogether, these mechanisms contribute to a reduced $CD4^+$ T cell recovery in particular in the naïve $CD4^+$ T cell subsets, causing a skewing of the naïve/memory ratio.

On the other side, various factors can generate a continuous depletion of $CD4^+$ T cells in INR patients. Increased death of $CD4^+$ T cells, in particular intrinsic apoptosis is reported and linked to $CD4^+$ T cell activation [323, 324, 344]. Recently, a small study found higher levels of anti-CD4 IgG in INR, capable of inducing $CD4^+$ T cell cytolysis and apoptosis *in vitro* [345]. Moreover, another report detected elevated expression of caspase-1 in INR indicating that also pyroptosis could contribute to the $CD4^+$ T cell loss [346].

Persistent T cell activation and cycling especially of $CD4^+$ T cells, as well as inflammation markers are found in higher levels in INR than immune responders in several studies [320, 324, 325, 334, 344]. Increased fractions of $CD4^+$ T cells expressing the exhaustion marker PD-1 has also been seen [320, 321, 344, 347]. In addition, the immune activation is associated with lymphoid fibrose [341] and fewer hematopoietic progenitor cells [114], and can thereby affect the $CD4^+$ T cell production as well.

Microbial translocation has been hypothesized to contribute to drive the chronic immune activation in INR. There are signs of gut dysbiosis, intestinal epithelial cell damage and break in gut junctions in several small studies of INR patients [171, 176, 177, 322, 348], as well as decreased gut epithelial cell proliferation and increased neutrophil infiltration [154]. Moreover, recent findings demonstrated that low nadir CD4 counts predicted gut dysbiosis and enterocyte damage, and INR status was associated with low microbial richness that correlated with systemic inflammation [172]. Inverse correlations between plasma levels of microbial products and $CD4^+$ T cell recovery support this theory [67, 71], although only some of the studies analyzing LPS in patients with impaired CD4 restoration have found increased levels [324, 349].

Despite long-term ART, residual HIV viremia can be detected in many patients by ultrasensitive assays [350]. Primarily, it probably origins from reactivation of long-lived, latently infected $CD4^+$ T cells (HIV reservoir) [93, 94], but ongoing viral replication in anatomical sites where ART is less consentrated also gives rise to sporadic detectable HIV RNA in the blood and fuels the HIV reservoir [93, 156, 351]. During ART, the size of the reservoir is related to both systemic immune activation and exhaustion, as well as $CD4^+$ T cell depletion [94, 95], indicating role of HIV persistence in INR. In support of this, intermittent low-level viremia above 20 copies/mL has been associated with reduced CD4 gain [352].

In addition, other chronic viral infections influence the immune status. Strong CMV specific T cell responses are associated with poor $CD4^+$ T cell recovery [329, 353] and asymptomatic CMV replication has been proposed as an important driver of the increased immune activation in INR [100]. Eight weeks of intervention with valganciclovir reduced T cell activation, but did not improve the CD4 count [100]. Association of HCV with incomplete $CD4^+$ T cell restoration and T cell activation have been demonstrated in several studies [103, 308, 354], and eradication of HCV in HIV/HCV co-infected reduced immune activation, microbial translocation and pro-viral DNA levels, although no effect on the CD4 count was shown [101, 103].

Moreover, disturbances in the Tregs pool apparently have impact on both the immune activation, T cell proliferation and recovery (see section 1.5.6), and seem to be present in higher fractions and more activated forms in INR [139, 143, 324, 355]. Yet, their precise role in the CD4 regeneration remains to be defined and sparse data exists on Treg subsets in INR.

Lifestyle factors such as smoking, alcohol abuse and obesity probably also contribute to increased immune activation in PLWH on ART, but are not directly connected to impaired $CD4^+$ T cell gain [76].

Genetic variations affecting immune recovery are a complex field. Variations in genes encoding IL-2, IL-2Rβ, IL-2Rγ, IL-15, IL15Rα, TRAIL, Bim, tumor necrosis factor

(TNF)α, and IFNγ and variations in CCR5 genotype and CCL3L1 gene copy number have been reported to influence $CD4^+$ T cell gain [356, 357].

In conclusion, incomplete immune restoration is strongly linked to a more advanced HIV infection before initiating ART. This is a situation characterized by a widely disseminated HIV reservoir, impaired gut immunity with a disrupted gut barrier and dysbiosis and consequently diverse sources that trigger immune activation and inflammation. Coexistent increased lymphoid fibrosis and higher age restrict lymphopoiesis. Nevertheless, some people with advanced HIV infection restore their CD4 count, whereas others with relatively high CD4 count pre-ART have a minor response to ART, indicating that the mechanisms influencing CD4 recovery are not fully uncovered and need to be further explored.

Table I. Mechanisms contributing to immunological non-response.

Etiology			
Decreased production of CD4⁺ T cells	Decreased hematopoiesis	Advanced HIV disease pre-ART	
		Residual HIV replication	HIV reservoirs
			Chronic immune activation / inflammation
			Reduced ART penetration
		Chronic immune activation / inflammation	Microbial translocation
			Co-infections
			Life-style factors
			Residual HIV replication
	Decreased thymopoiesis	Age	
		IL-7	Reduced IL-7 receptor expression and IL-7 response
	Reduced peripheral T cell renewal	Lymphoid fibrosis	Advanced HIV disease pre-ART
			Residual HIV replication
			Chronic immune activation / inflammation
		T cell exhaustion	Chronic immune activation / inflammation
			Residual HIV replication
		IL-7	Reduced IL-7 receptor expression and IL-7 response
			Disrupted lymphoid architecture
Increased destruction of CD4⁺ T cells	Increased chronic immune activation / inflammation	Microbial translocation	Dysbiosis and disrupted gut barrier and immunity
		Co-infections	CMV, HCV
		Life-style factors	Smoking, alcohol, obesity
		Residual HIV replication	HIV reservoirs
			Chronic immune activation / inflammation
			Reduced ART penetration
	Pyroptosis	Incomplete HIV reverse transcription	
Genetic variations			

1.6.3.2 Clinical consequences of INR

An incomplete immune response leads to unfavorable prognostic implications for the patients. It is widely documented that a lower CD4 count on ART is associated with higher future risk of developing AIDS [315, 319, 358], non-AIDS morbidity [298, 358-360] or mortality [109, 292, 298, 319, 358, 359, 361]. The life expectancy seems to decrease with decreasing CD4 counts [288]. Those who fail to achieve CD4 count to > 200 cells/µL are at the highest risk, but also INR with CD4 counts 200-350 cells/µL are shown to have higher incidence of AIDS-defining illnesses [311, 315], non-AIDS related malignancies [362], cardiovascular disease [360] and mortality [292, 363], highlighting the importance of completely restoring the CD4 count.

1.6.3.3 CD4/CD8 ratio

In addition to low CD4 restoration as an adverse prognostic marker, there is increasing evidence for an association between low CD4/CD8 ratio and T cell activation, senescence, inflammation markers [364], low functional impairment [110] and higher non-AIDS morbidity [365, 366] and mortality [364]. Conflicting data exist whether CD4/CD8 ratio baseline predicts non-AIDS mortality in those with high CD4 count [367, 368]. In the modern ART era only about 1/3 will achieve a normalized CD4/CD8 ratio >1 after long-term ART [366, 369, 370] and most of these patients concomitantly restore their CD4 count to >500 cells/µL [369, 370]. Starting ART during primary HIV infection, at higher CD4 counts, or with low $CD8^+$ T cell numbers have been shown to increase the likelihood of acquiring a CD4/CD8 ratio >1 [364, 366, 369], whereas CMV seropositivity are reported to affect the ratio negatively [366, 370].

1.6.4 Therapeutic possibilities in INR

Given the substantial evidence of persistent chronic inflammation in treated HIV infection, different therapeutic strategies to reduce this inflammation have been a main focus of interest during the recent years. Various interventions have also been tested in attempt to increase the CD4 count in INR.

1.6.4.1 Early ART

Early ART start, particularly during the acute phase of HIV infection, is probably our best tool in order to further improve outcome for PLWH. Enhanced immune restoration and reduced chronic immune activation have been shown, although unfortunately the chronic inflammation does not seem to be abrogated. Studies have demonstrated decreased HIV-reservoirs [371-373] and improved gut mucosal immune function [65, 150]. Immediate ART decreased $CD8^+$ T cell activation, D-dimer and TNF to levels similar to high-risk uninfected controls. CRP and sCD14 were not normalized, but reduced compared to individuals who started ART during chronic phase [65, 374]. A higher proportion of individuals was reported to reach a CD4 count above 900 cells/μL by ART initiation within four months after seroconversion vs a modest deferral [304], and also by ART start within the first year compared to those who started somewhat later [310]. Fewer developed AIDS in the group who commenced ART within 12 months after seroconversion [304, 310].

ART intensification with use of raltegravir and/or maraviroc both in acute HIV and during chronic infection have shown inconsistent results and no clear immunological, virologic or clinical benefit [375, 376], not even in patients with incomplete immune recovery [377-380]. Yet, this cannot be recommended as strategy to reduce immune activation or improve CD4 gain.

1.6.4.2 Immune modulating strategies

In general, therapeutic intervention studies in INR have been disappointing. The best documented treatment being recombinant (rh) IL-2 therapy in addition to ART. Despite increased CD4 count, no clinical benefit during long-term follow-up has been seen. This was probably due to substantial expansion of the Tregs pool [381, 382]. Perhaps the most interesting studies the recent years have been rhIL-7 supplement to virally suppressed PLWH with CD4 count <350-400 cells/μL in the INSPIRE 2 and 3 trials. rhIL-7 increased the CD4 count, but repeated cycles were required for most participants [383]. On the other hand, there was a high rate of blips and an expansion in the HIV reservoir probably caused by both cell proliferation and neo-infection [383, 384]. Thus, long-term effects with regards to both CD4 count, inflammation markers, HIV reservoir

and clinical endpoints remain to be determined. Piconi *et al* demonstrated both reduced innate and adaptive immune activation following six months of 400mg hydroxychloroquine to INR with CD4 count <200 cells/μL on stable ART [385]. However, the number of patients was low, no control group was included and other studies with hydroxychloroquine intervention have shown conflicting results [386]. Alternative intervention strategies are currently under investigation, for instance a pilot trial with PD-1 blockade in order to reduce T cell exhaustion is now being investigated in PLWH with low CD4 recovery on ART (Clinical Trials.Gov NCT03367754).

Treatment of co-infections and use of anti-inflammatory agents are other approaches in order to reduce chronic inflammation and improve outcome in virally suppressed PWLH. Most studies have been performed in patients with high CD4 counts and show few beneficial effects. 24 weeks with rosuvastatin reduced sCD14 and the fraction of TF positive non-classical monocytes independently of decreased lipid levels [387]. The REPRIVE study (NCT02344290) addresses the impact of statin intervention on clinical endpoints in a large group of ART-treated. IP-10 levels decreased significantly during 16 weeks of sitagliptin treatment, but returned to baseline levels soon after drug discontinuation and the reduction was not accompanied by changes in other inflammation markers or cell subsets [388]. Neither 12 weeks of aspirin, nor 24 weeks of low dose methotrexate intervention showed any effect on soluble inflammation markers or endothelial dysfunction compared to placebo [389, 390]. People in the low dose methotrexate group experienced more safety events. Furthermore, a pilot study with addition of a PAR-1 antagonist for 12 weeks to target coagulopathy and inflammation in virally suppressed with high D-dimer failed to reduce D-dimer and other soluble inflammation markers [391]. Use of angiotensin converting enzyme inhibitor or angiotensin II receptor antagonist as anti-fibrotic agents in two small trials revealed no effect on fibrosis in rectal lymphoid tissue or lymph nodes [392, 393]. In addition, several anti-coagulation and immune-based therapies such as inhibition of FXa (NCT02339415), IL-6 (NCT02049437) and IL-1β (NCT02272946) are ongoing. Hence, currently, there is lack of sufficient evidence to recommend any specific intervention to improve CD4 count or reduce inflammation in PLWH on ART.

2. Objective of the study

The overall objective of this thesis was to characterize chronic immune activation and inflammation in virally suppressed PLWH on ART, but without a normalized CD4 count, so-called immunological non-responders (INR), to search for biomarkers for immunological non-response, and to evaluate the effects of probiotic intervention in virally suppressed PLWH with a subnormal CD4 count.

The specific aims were:

Paper I: To investigate the effects of eight weeks of intervention with a multistrain probiotic in a cohort of ART-treated and virally suppressed PLWH with a subnormal CD4 count:

- Primary endpoints:
  i. Safety and tolerability
  ii. Microbial translocation
  iii. T cell activation
- Secondary endpoints:
  i. Immune reconstitution (CD4 count)
  ii. Gut microbiota composition
- Exploratory endpoints:
  i. Markers of inflammation
  ii. Lymphocyte phenotypes

Paper II: To compare and characterize T cell activation, Tregs subsets, microbial translocation and soluble inflammation markers in INR and immunological responder (IR) cohorts in order to identify possible biomarkers associated with a discordant response to ART.

Paper III: To compare and characterize innate immunity in INR and IR cohorts by analyzing the distribution, activation level and *in vitro* IP-10 and IDO responses in monocyte and DC subsets, and to explore their association with future CD4 recovery in INR.

3. Summary of the papers

3.1 Paper I

This study explored the impact of probiotic intervention on microbial translocation and inflammation in PLWH on ART with viral suppression and CD4 count < 500 cells/µL. Study participants were randomized in a double-blinded fashion to daily multistrain probiotic containing *Lactobacilli* and *Bifidobacteria* (n = 15), placebo (n = 9), or controls (n = 8) for eight weeks.

Twenty-four participants completed the study and were included in analyses of soluble inflammation markers, T cell activation and gut microbiota composition. The probiotics were well-tolerated and no serious adverse advents were recorded. In patients receiving probiotics (n=11) there was a significant reduction in D-dimer levels and a tendency to reduced levels of CRP and IL-6. Fractions of *Bifidobacteria* and *Lactobacilli* increased in the probiotic group, whereas the relative abundance of *Bacteroides* decreased. There were no significant changes in markers of microbial translocation or T cell activation and the CD4 counts in the intervention group were stable.

This study was one of the first to target the gut microbiota and to analyze deep sequencing data in PLWH on ART with a subnormal CD4 count, and the results indicate that probiotic intervention might have a beneficial effect upon inflammation in these patients. However, the findings should be elaborated in lager studies with long-term follow-up.

3.2 Paper II

This study explored patterns in T cell phenotypes, microbial translocation and inflammation in order to identify biomarkers associated with an inadequate immune recovery. Forty-one INR with CD4 count < 400 cells/µL and 26 immune responders

(IR) with CD4 count > 600 cells/μL were included. CD4 counts were also registered two years after inclusion.

In the INR cohort the plasma IP-10 levels were higher, the T cells were more activated ($CD38^{+}HLADR^{+}$), the naive/effector memory (EM) T cell ratio was lower and the proportion of resting Tregs ($CD4^{+}CD45RA^{+}FoxP3^{+}$) was reduced compared with the IR group. There were no differences in markers of microbial translocation between the groups. INR patients with CD4 counts ≤ 300 cells/μL also showed a higher fraction of activated Tregs (aTregs) defined by $CD4^{+}CD147^{high}CD25^{high}$, and in these patients IP-10 levels and the kynurenine/tryptophan ratio were negatively associated with the CD4 counts two years after inclusion.

The study presents for the first time data on the $CD147^{high}CD25^{high}$ aTreg subsets in PLWH. Overall, our findings contribute to the understanding of the immunological mechanisms causing an inadequate CD4 increase in INR patients and suggest IP-10 as biomarker for INR.

3.3 Paper III

This study explores the function and characteristics of innate immunity in INR by studying differences in monocyte and DC activation and *in vitro* responses, and their relation with future CD4 gain. The included participants were the same cohorts as in paper II. Ten ART-naive (never treated) PLWH and 10 HIV-negative healthy persons served as controls. CD4 counts were also registered after two and four years. Intracellular IP-10 and IDO in monocytes and DCs and cell culture supernatant cytokines were measured in *in vitro* aldrithiol-2-inactivated (AT-2) HIV-1 stimulated PBMC.

The INR cohort displayed higher spontaneous activation of both monocytes (HLA-DR), mDCs and pDCs (HLA-DR, CD83 and CD86) compared with IR, findings that were associated with T cell activation, an EM T cell phenotype and more activated Tregs. There were no differences observed between the groups in IP-10, IDO or cytokine

responses after *in vitro* HIV-stimulation, although in INR, both IP-10, IDO and cytokine upregulation were inversely correlated with the fraction of aTregs. A high *in vitro* HIV-induced intracellular IP-10 expression in monocytes was related to a lower prospective CD4 gain after both two and four years.

This is the first study presenting thorough characterization of activation and functionality of innate immune cells and their relation with T cell phenotypes in an INR cohort. Activation of monocytes and subsequent IP-10 expression could be a relevant pathway involved in inflammation and poor immune reconstitution in PLWH on ART.

4. Methodological considerations

4.1 Study design, patient selection and ethical considerations

The study participants were HIV-infected adults (> 18 years) recruited from the outpatient clinic at Department of Infectious Diseases at Oslo University Hospital (OUS), Ullevaal. In **paper I** (Clincal Trials.Gov NCT01439841), eight of the patients were included at Karolinska University Hospital. The healthy controls were health care workers who volunteered to donate samples. All participants provided written informed consents and the three studies were approved by the Regional Ethics Committee at Oslo University Hospital (2011/1449 C, 1.2007.83 and 2015/629), and for paper I also by the Regional Ethics Committee at Karolinska University Hospital (2012/459-31/3). Table II gives an overview of the study designs, the patients included and specimen obtained in the different studies.

Paper II and III comprise the same cohort. Inclusion criteria were continuous ART for minimum 24 months, with full viral suppression for the last 18 months together with CD4 count and age criteria as mentioned above and in table II. The study participants were selected from the HIV cohort quality database based on the inclusion criteria. They received an invitation letter to participate in English or Norwegian, followed by a phone call with further information about the study. 84 patients showed interest and attended for blood-sampling, a thorough clinical examination and review of their entire HIV and medical history. Participants with concomitant or sporadic use of immune modulation therapies, acute or chronic severe illnesses, including opportunistic infections, autoimmune diseases, active chronic hepatitis B (HBV) and HCV, cancer or TBC were excluded. There were none IDU. The cohorts were balanced with regard to risk groups with 50% MSM and 50% from other risk groups and 65-70% with Caucasian ethnicity, reflecting the population at our outpatient clinic. Information about the invited patients who declined to attend should have been registered in order to clarify if there was a skewed selection of participants. A recent review of the total INR cohort at our outpatient clinic showed a slightly higher proportion of women (24%), a shift in risk

groups with 11% IDU and 36% MSM, and 58% with Caucasian ethnicity. Furthermore, higher number of immunological responder (IR) participants would have been an advantage as it could have increased the statistical power [394].

Table II. Study design, participants and obtained specimen in paper I-III.

	Paper I	**Paper II**	**Paper III**
Design	Randomized, placebo-controlled, double-blinded	Cross-sectional[a]	Cross-sectional[b]
Inclusion site	OUS, Karolinska	OUS	OUS
Inclusion criteria			
Duration of ART, mo	> 6	≥ 24	≥ 24
Duration of viral suppression, mo	> 6[c]	≥ 18[d]	≥ 18[d]
Study participants, inclusion criteria			
ART+, CD4 < 500 cells/µL	32		
INR: ART+, CD4 < 400 cells/µL		41	41
IR: ART+, CD4 > 600 cells/µL		26	26
ART naive[e]			10
Healthy controls			10
Specimen	Plasma, serum, PBMC, fecal samples	Plasma, PBMC	Plasma, PBMC

[a] CD4 count also registered 2 years after inclusion
[b] CD4 count also registered 2 and 4 years after inclusion
[c] HIV RNA < 50 copies/mL
[d] HIV RNA ≤ 20 copies/mL
[e] Duration of HIV infection ≥ 1 year
Mo, months.
OUS, Oslo University Hospital

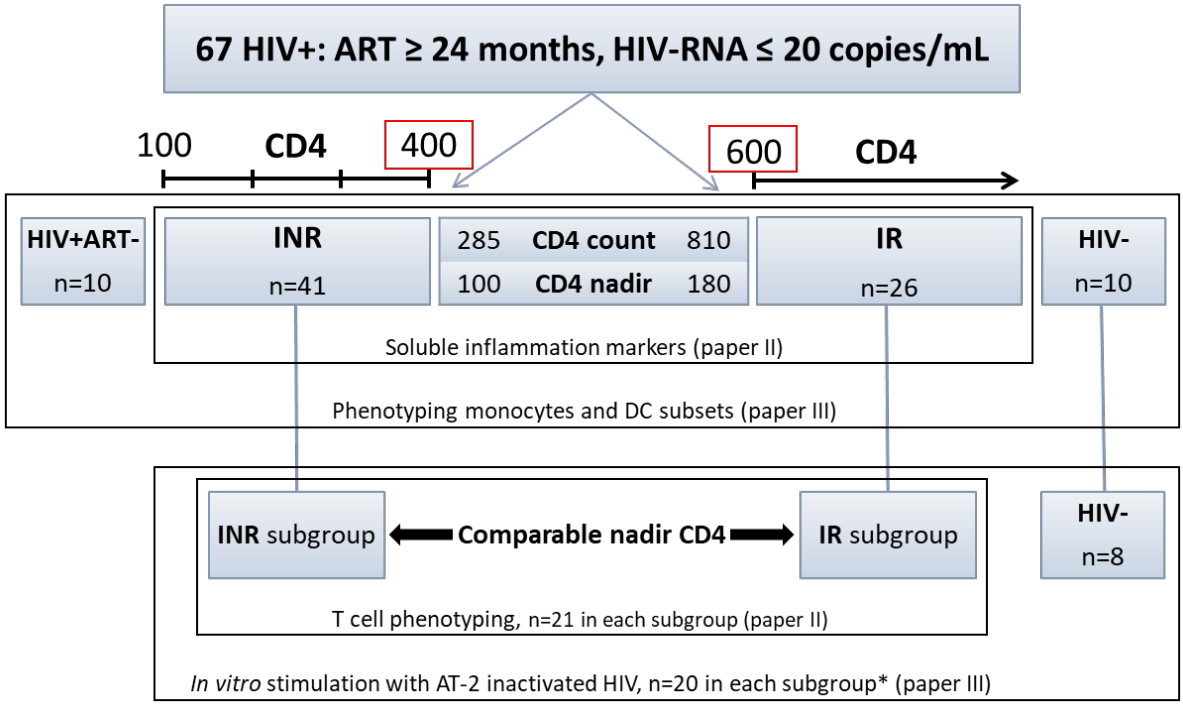
For comparison in **paper III**, age and gender matched ART-naïve (never treated) PLWH and HIV negative healthy controls were recruited; the ART-naïve either from the Deparment's biobank or consecutively from our HIV outpatient clinic. A more optimal healthy control group would have been HIV-negative persons with similar demography, ethnicity and lifestyle as the HIV cohorts, minimizing confounders. The reason for including these control groups was to determine the various parameters in untreated HIV infection as well as in healthy individuals. However, the main purpose with this study was to compare INR with IR.

This was a cross-sectional study, and the results therefore demonstrate associations and not causality. Still, CD4 counts were obtained prospectively from the HIV cohort quality database after approximately two years for **paper II** and also after four years for **paper III**.

4.1.1 Definition of INR

As previously decribed, there is no universal definition of INR status. Perez-Santiago *et al* recently concluded that a CD4 cutoff value of 400 cells/µL best separated INR and IR according to opposite immunological phenotypes rather than other cutoffs, or different CD4 increments from nadir [321]. Taking into account the knowledge of the slope of the CD4 increase that declines 3-4 years after ART start [301, 305-307], including only patients with ART-duration for more than four years could be argued. However, several studies of INR use almost similar criteria with viral suppression for a minimum of 24 months [139, 321, 323, 325]. In addition, 75% of the included INR patients in our study had been under continuous ART for more than three years. Lederman *et al* also showed a much steeper increase of the CD4 slope in immune responders compared to INR the first couple of years after ART initiation, supporting an early differentiation between these patient groups [325]. After median 4.7 years follow-up, only 37% of our INR patients achieved a CD4 count above 400 cells/µL and 8% above 500 cells/µL. These numbers are in line with other studies [321] and show that very few in this INR cohort reached an acceptable CD4 count even after long-term ART.

The main reason for defining IR with CD4 count above 600 cells/µL was to ensure good separation between the groups in order to detect possible minor differences in immune profile and functionality.



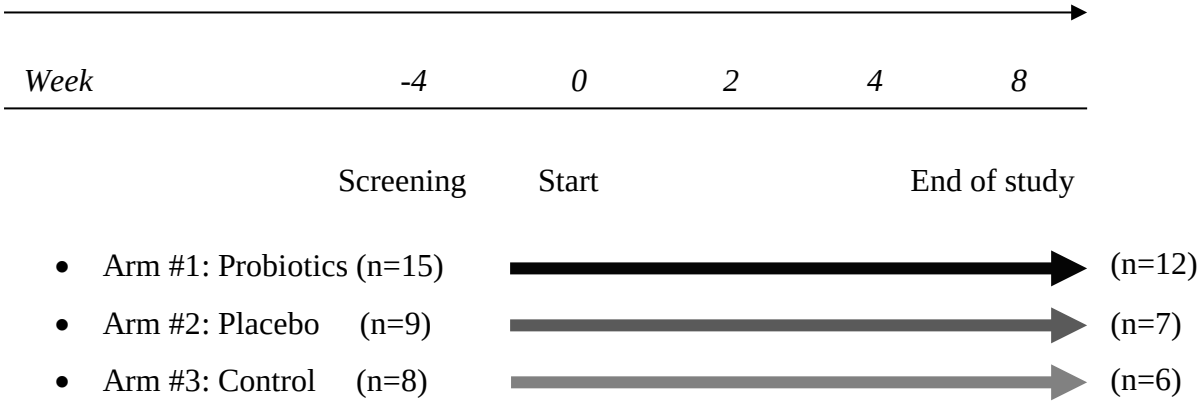
* Due to shortage of cells, 4 INR and 2 IR patients from the subgroups in paper II were not included in paper III. In paper III, 3 other INR and 1 other IR patients were thus retrieved from the main INR and IR cohorts for the assays with *in vitro* stimulation with AT-2 inactivated HIV.

Figure 13. Overview of the included participants to the different analyses in paper II and III.

4.1.2 Study outline, randomization and blinding in paper I

Randomized controlled trials are often the preferred design for studying efficacy of healthcare interventions as randomization reduces selection bias [395, 396]. If proper randomization is conducted, any observed differences in the baseline characteristics will have occurred by chance. In **paper I** the study nurse consecutively searched for eligible patients from the outpatient clinic. The study participants were randomized in a double-blinded 2:1:1 fashion to probiotics, placebo and controls, respectively. The reason for including a control group in addition to the placebo group was the potential for microbial products having biological effects despite heat treatment. The randomization was performed in blocks of eight using a predefined list of random digits followed by drawing of opaque, sealed envelopes from an urn and the randomization list was kept locked in a separate office.

Table III. Study outline for paper I.



Week	**-4**	**0**	**2**	**4**	**8**
	Screening	**Trial period**			
Informed consent	**x**				
Inclusion/exclusion	**x**				
Randomization		**x**			
Adverse events			**x** (phone)	**x**	**x**
Supply (Arm #1 & 2)		**x**		**x**	
Physical examination		**x**			**x**
Blood sample		**x**		**x**	**x**
Fecal sample		**x**			**x**

Too ensure blinding of the intervention, the probiotics and the placebo were produced with equal taste, packed in neutral packaging and marked with study and visit number. Furthermore, participants, healthcare providers and the investigators were blinded for the type of intervention throughout the study period and data collection. The untreated control arm was open. External collaborators performing the laboratory analyses of D-dimer, CRP, tryptophan catabolism and microbiota analyses did not have access to the randomization code, which was broken prior to statistical analyses. This double-blinding should usually contribute to unbiased follow-up of the groups, lower discontinuation of the study participants, as well as decrease biased physical and

psychological responses to the intervention and reduce differential assessment of outcomes [397]. The unmasking of the blinding prior to statistical analyses could potentially have influenced generation of the results, however most of the outcomes in this study were quantitative laboratory values leaving less opportunity for bias.

This trial was considered a phase II exploratory study with unknown outcome estimates and variations, and for that reason, no formal sample size calculations were performed. In exploratory intervention trials safety is a primary outcome. The number of patients should therefore be kept to a minimum, but at the same time be sufficient to provide reliable conclusions. Based on comparable intervention studies [398, 399], we aimed to include 50 study participants. However, we experienced a relatively slow inclusion rate and a demanding and costly production and distribution line of the dairy-based probiotics and placebo products. Hence, only 32 participants were recruited during the settled period for fresh-made probiotics production, and among them 78% completed leaving a relatively small study population, which could have biased the outcome (see statistical considerations 4.6). The drop-out rate was evenly distributed between the randomized groups and should not systematically skew the results.

4.2 Sample processing and storage

4.2.1 Plasma and serum samples

Citrat and EDTA plasma were snap frozen in pyrogen-free tubes, centrifuged within 20 minutes at 2000g and 4°C for 20 minutes, and serum was centrifuged after coagulation in room temperature. All samples were stored at -80°C in a biobank and analyzed in batches. This simplifies logistics and reduces inter-assay variability. Long-term storage even in -80°C and freeze-thaw circles can lead to degradation of cytokines and chemokines, some more vulnerable than others [400-402]. However, our analyses were run within two years, except for IP-10 and tryptophan in **paper I** and multiplex in **paper II**, which were analyzed within 2.5 years. Furthermore, most of the assays were performed on previously unthawed plasma with some few

exceptions carried out in plasma thawed only once before.

4.2.2 Cryopreserved cellular samples

For all flow cytometry experiments we used peripheral blood mononuclear cells (PBMC) isolated in cell preparation tubes (CPT) with sodium heparin. CPT separates mononuclear cells from whole blood using a liquid density medium and an inert gel barrier, which after centrifugation separates the PBMC in plasma at the top of the tube and the erythrocytes and granulocytes in the bottom. The PBMC was aliquoted, cryopreserved and stored at -150°C until thawing and analyses in batches. Each of these steps can cause variability in the assay results and standardization of the procedures is therefore important.

The processes associated with cryopreservation can induce functional and phenotypical changes in PBMC and could in particular reduce the fractions of pDC [403] and Tregs [404, 405]. Still, for T cell phenotyping the correlations between fresh and frozen samples are high [406], DC subsets do not seem to be altered phenotypically [407] and a main advantage is that batch analyses on cryopreserved samples improve comparability of data. Rapid thawing in 37°C water-bath and dropwise adding of preheated medium is shown to enhance viability [406, 408, 409] and was conducted for the flow cytometry experiments in **paper I and III**.

Overnight resting in horizontal position and concentration of cells between 2-5 $x10^6$ cells /mL, is recommended for immune-phenotyping of lymphocytes as it reduces number of dying cells and improve quantity and quality of the T cells [410, 411]. This was ensured for the T cell phenotyping in **paper I** and the cell viability exceeded 85%. The T cell phenotyping in **paper II** was performed at another laboratory with a more rapid thawing protocol and without resting, which probably contributed to a lower viability, although none or short resting times seem to give high viability when the processing of the CPT starts within a few hours [412].

Nevertheless, for monocytes, resting can reduce the number of these cell types [410]. Furthermore, already after six hours without stimulation, there seem to be a transition

from CD14++CD16- monocytes to CD16+ monocytes in whole-blood [413]. Based on these findings, thawed and washed PBMC were left resting for only two hours in the monocyte and DC experiments (**paper III**).

4.2.3 Feces samples

Stool samples represent the most convenient sample collection method for gut microbiota samples and were collected from study participants in **paper I**. Fecal samples can also be obtained from luminal brushing and mucosal biopsies giving information of the microbiome living in closer contact with the gut immune system at specific sites in the gastrointestinal tractus. However, these methods are more invasive and bowel cleansing before mucosal biopsies can probably affect the microbiota of these samples [414]. Feces was sampled without preservatives, kept cold until study visit, frozen within 24 hours and stored at -80° C until further analysis.

4.3 Analysis of circulating markers

4.3.1 ELISA

The enzyme-linked immunosorbent assay (ELISA) can quantify or test for presence of an antigen or ligand with high sensitivity [415]. It is highly specific, easy to conduct, relatively inexpensive and therefore widely used [415]. Sandwich ELISA use plastic microplates pre-coated with monoclonal antibodies specific for the actual antigen. Following a solution containing animal serum to prevent non-specific binding of the analyte, standards and samples containing soluble antigens are added to the wells and will then bind to the solid phase reactant. Addition of an enzyme-coupled antibody specific for the antigen will lead to color development of a supplemented substrate proportionally to the amount of bound antigen. The reaction is stopped after a specific interval and the color intensity measured by an ELISA reader. During the process washing steps are performed to remove unbound reagents. The concentration of the present antigen in the samples is determined by comparing light absorbance in the

assay well with a standard curve obtained by analyzing different known concentrations of the analyte [415].

Non-specific binding can give false positive results and is a general limitation of ELISA. Furthermore, the concentration of reagent being quantitated should be within the dynamic range of the standard curve [415]. The serum and EDTA plasma samples were therefore diluted for analyses of IP-10 and sCD14, respectively. To increase validity of the results, all the samples were run in batches and in duplicates. The different patient groups were equally spread on two microtiter plates for every analysis, and for **paper I** pre-and post-intervention samples were run on the same plate. The intra-assay CV% was calculated by the mean of all CV% on one plate and was <8%. One, two or three longitudinal samples run in duplicates were used to calculate inter-assay CV% and the inter-assay CV% was generally acceptable and <13%. More specific, for the hs-IL-6 and D-dimer analyses in **paper I** and IP-10 in **paper II**, the intra-assay and inter-assay CV% was <5% and <9%, respectively. However, a higher number of longitudinal controls would have given a more precise inter-assay CV%.

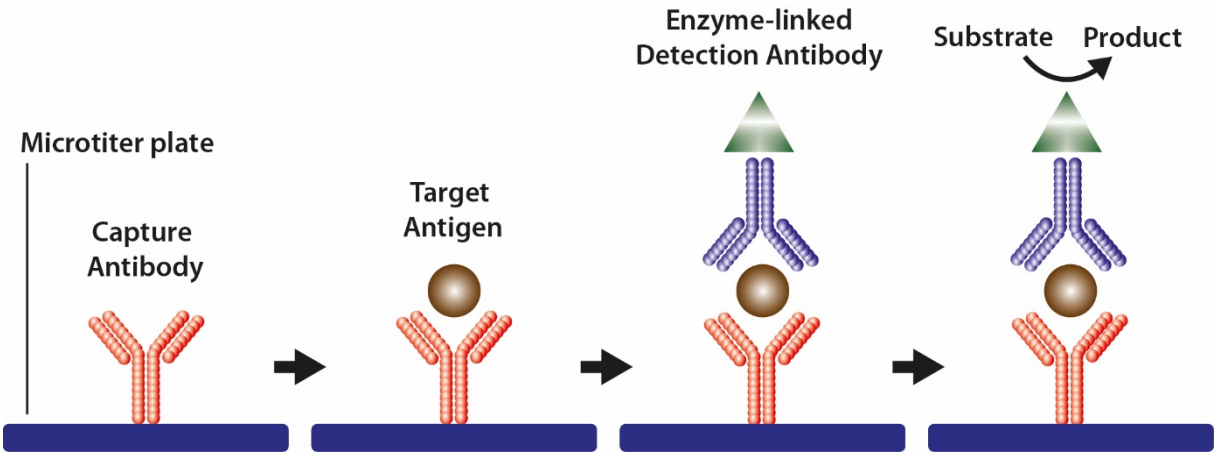


Figure 14. Schematic presentation of ELISA principle.

Modified by permission from Springer Nature: Nature Nanotechnology. Plasmonic ELISA for the ultrasensitive detection of disease biomarkers with the naked eye, Roberto de la Rica, Molly M. Stevens, 2012. (License number: 0459887110210).

4.3.1.1 The Limulus amebocyte lysate (LAL) colorimetric assay

The LAL assay quantifies levels of Gram-negative bacterial endotoxin (LPS). LPS catalyzes the activation of a proenzyme in the LAL derived from blood cells in horseshoe crabs. The rate of this activation is proportionate to the concentration of present LPS. Next, the activated enzyme cleaves a chromophore from an added synthetic substrate, producing a yellow color. The reaction is stopped with a stop reagent and the concentration of LPS in a sample is calculated from the absorbance values of solutions containing known amounts of endotoxin standards [416].

This assay is being widely used, but still has several potential pitfalls making the inter-assay variations higher than standard sandwich ELSA and comparison of levels between studies is challenging. It is highly sensitive to LPS [417] and careful handling of all equipment, samples and reagents is necessary to avoid contamination and a high background. Pyrogen-free, sterile materials were used and samples were diluted 10-fold to avoid interference with the background color. To abolish inhibitors of endotoxin activity in plasma and to dissolve immune complexes, preheating to 68°C for 12 minutes was performed prior to analyses. The intra-assay CV% of our LPS analyses were good, <5%, but the inter-assay variation more variable with means 8% and 16% in **paper I** and **II**, respectively.

4.3.2 Multiplex

In contrast to ELISA, microsphere-based multiplex immunoassays allow for simultaneous measurement of up to 100 analytes in a single patient sample and thereby reducing assay costs, time consumption and sample volumes [418]. Multiplex assays combine the principles from sandwich ELISA and flow cytometry. Antigen-specific capture antibodies attached to color-coded magnetic beads (known as microspheres) are utilized instead of microplates pre-coated with antibodies. The instrument combines two lasers and detects individual beads by flow cytometry; one laser for excitation and identification of the microsphere and another is used to excite and quantify the amount of target protein bound to the individual beads [419]. The signals are directed to detectors and concentrations of the analytes are determined by extrapolation from an

internal standard. Despite the dynamic assay range, outliers above or below the limits of detection are prevalent and dilutions of samples can be needed. In **paper II**, eight of the analyzed markers were excluded because of low plasma levels and for the remaining markers values below the lower detection limits (LDL) had to be replaced by a fixed value below LDL. Cross-reactivity between detection antibodies and specific targets can also represent a challenge [420]. Multiplex performed in **paper II** were run in singlets giving risk of more inaccurate data. Furthermore, storage time, freeze-thaw circles and anti-coagulants used in the plasma samples may affect the levels of cyto-and chemokines [421].

Concentration of cyto-and chemokines in cell-culture supernatants can be influenced by the distribution of the different cell-types in the PBMC, which possibly varied between the patient cohorts. The use of a cytokine secretion inhibitor also affects the cytokine concentrations, and a different timing of addition, or non-use of Brefeldin A could probably have given other supernatant cytokine results in **paper III**. Aldrithiol-2-inactivated (AT-2) HIV stimulated pDCs have been shown to produce IFNα after 4 hours, but in higher amounts between 6 and 12 hours [218] and stimulation of PBMC with AT-2 HIV in equivalent concentration and time without cytokine secretion inhibitor yielded considerably higher concentration of INFα than observed in our experiments [221]. Use of another detection assay could also have influenced the IFNα2 levels.

4.3.3 Liquid chromatography – tandem mass spectrometry

Liquid chromatography-tandem mass spectrometry (LC-MS) was used to quantify tryptophan metabolites and neopterin in plasma in **paper I** and **II.** Compared to immunoassays, LC-MS is more specific and highly sensitive [422]. It has a wide dynamic range and allows for analyzing multiple components simultaneously using small sample volumes with minimal sample preparation [422]. LC-MS couples high resolution chromatographic separation with sensitive and specific mass spectrum detection. First, the compounds in a sample are separated by liquid chromatography utilizing a stationary and mobile phase. The analytes are then ionized and ions are separated and characterized based on their charge/mass ratio by using two mass

spectrometers in series. The actual biomarkers analyzed by LC-MS are reported to be stable during storage and freezing [423].

4.3.4 Calibrated automated thrombogram

The observed reduction of D-dimer after probiotic intervention in **paper I** made further study of coagulation relevant. Calibrated automated thrombogram measures thrombin generation in clotted plasma. Excessive thrombin formation always causes a thrombotic tendency [424] and thrombin production reflects the majority of thrombotic-hemostatic mechanisms in the blood [425]. Coagulation is triggered *in vitro* by platelet-poor-reagent containing 5pM Tissue Factor and calcium. By using a chromogenic substrate, signals are measured and the thrombin formation can subsequently be calculated [425]. The generated thrombogram describes the course of the thrombin formation. Initially no thrombin is formed (Lagtime), followed by a steep increase of the thrombin concentration to a peak (Peak thrombin) and finally it declines. The AUC reflects the endogenous thrombin potential. These parameters were obtained and then normalized to a plasma pool of healthy controls and presented in percent. The samples were run in duplicates and the inter-assay CV% was <10%. Based on the knowledge that thrombin generation in platelet poor plasma is reduced with anticoagulant treatment, two individuals using warfarin were excluded from these analyses [425].

4.4 Flow cytometry

4.4.1 Basic principles in flow cytometry

Flow cytometry technology has been of major importance in HIV-research from the very beginning and an essential tool for characterization of the immune responses following HIV infection and treatment, as well as understanding the biology of the virus [426]. Flow cytometry is a laser-based technology used to analyze physical and chemical characteristics of cells or particles in a fluid. Cell size, granularity, counts, surface receptors, intracellular proteins, nucleic acid content, enzyme activity and cell cycle

properties can among others be identified. One of the main advantages of flow cytometry is the ability to measure several properties at a single cell level.

The first step is to stain the epitope of interest with specific fluorochrome-conjugated monoclonal antibodies. Fluorochromes are excited by lasers at a given wavelength and re-emit (emission) lights at a longer wavelength with less energy, which are then detected.

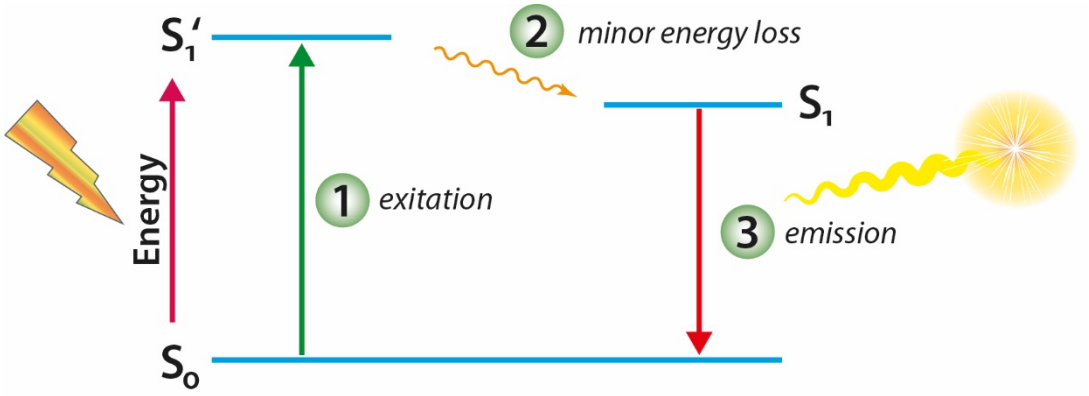


Figure 15. Diagram of the fluorescence process.

Modified from Exyte expert cytometry.

A flow cytometer consists of three main systems: fluidics, optics and an electronic system. The fluidics system moves the cells precisely one by one through the interrogation point where the cells are illuminated by the light from the lasers. Passage of single cells is generated by hydrodynamic focusing by a laminar sheath flow. Two types of light are measured as particles pass through the illumination source; i) light scattering gives information about the particle's size through the forward scatter and granularity by the side scatter. ii) Fluorescence emission can provide quantitative and qualitative data about fluorochrome-labeled epitopes. The emitted light is then directed to the different detectors by mirrors and optical filters that isolate the desired wavelength bands. When light hits a photodetector / photomultiplier tube (PMT) a small current is generated. This voltage is amplified into lager electrical signals and finally converted into digital values available for subsequent data processing [427].

We performed the flow cytometry experiments for **paper I and III** on a 9-channel BD FACS Canto II and used a 12-channel BD LSR Fortessa for **paper II**.

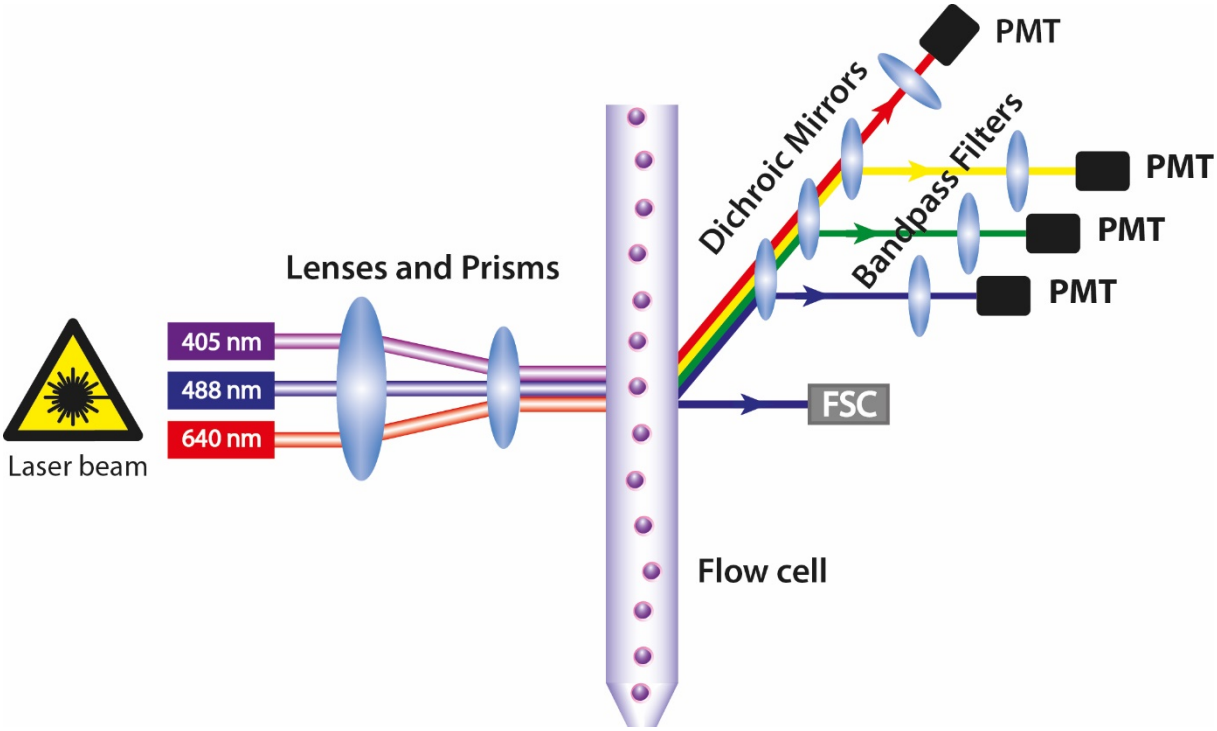


Figure 16. Schematic diagram of a flow cytometer. Fluorochrome stained cells pass one by one through the flow cell and the interrogation point where they are excited by the lasers. Light scattered by the cells itself and emitted light are then directed to photomultiplier tubes (PMT) by mirrors and optical filters that isolate the desired wavelengths.

Modified by permission from Plos: Plos Computational Biology. Flow cytometry bioinformatics, Kieran O'Neill, Nima Aghaeepour, Josef Spidlen, Ryan Brinkman, 2013. (Open access, unrestricted use)

4.4.2 General challenges in flow cytometry analyses

When conducting flow cytometry experiments there are several challenges involving the choice of reagents, cell-preparation and optimization of the flow cytometer and data analyses. Dimmest fluorochromes should be used in combination with brightly staining antibodies while reserving the brightest fluorochromes for dimly staining antibodies [428]. See table I and II in the appendix for selection of the fluorochrome-conjugated antibodies used in the different assays.

To reduce impact of “drift in the flow cytometer” all flow cytometry experiments were analyzed in batches over a period of a few weeks, the monocyte and DC stimulation assays and phenotyping (**paper III**) were carried out within one and two weeks, respectively. Samples from the various study cohorts were equally distributed between the batches. Cytometer setup and BD tracking beads (CS&T) were performed every working day as a quality control in order to align the lasers and set the PMT amplification voltage.

Strategies to reduce background fluorescence

A main concern in flow cytometry experiments is to adequately separate positive and negative cell populations and this operation is negatively influenced by the background fluorescence. There are recommended strategies to reduce autoflourescence, spectral overlap and undesirable antibody binding, the three main causes of background fluorescence [429]. i) Dead cells have higher degree of autofluorescence and must therefore be excluded [427]. In **paper I and II** a strict lymphogate was set to eliminate dead cells which usually have lower size in the forward scatter [427], while fixable viability dye was used to gate viable cells in the experiments in **paper III**. ii) In multicolor flow cytometry experiments the spectra of the different fluorochromes may overlap and thereby creating spillover into a secondary detector, giving a false signal in the latter channel. Compensation is a process where spillover to all detectors from single-color stained samples is registered and the background is subsequently subtracted. We used unstained and single-color stained antibody capture beads when creating compensation controls. The compensation values were calculated from the software and was applied before data acquisition [430].

iii) Several operations can be carried out to reduce background caused by non-specific binding of antibodies. First, all antibodies in our experiments were titrated to determine its optimal concentration for staining. Titration is a process where the antibodies are serially diluted with the intention to find the concentration that gives the best separation of the positive and negative population [427, 429]. Furthermore, monocytes in particular, but also DCs have high abundance of Fc-receptors and in order to reduce the component of non-specific binding to Fc-receptors in the assays in **paper III**, the PBMC

were incubated with an Fc-block prior to staining with surface markers [431]. Finally, isotype controls determine non-specific staining of an antibody of a particular isotype conjugated to a particular fluorochrome (see below).

Gating controls

For best visualization of events against the axes, the bi-exponential display was used. A gating control is important for defining the boundaries when there is an indistinct division between positive and negative cell populations [432]. Fluorescence minus one (FMO) control, isotype controls and biological controls are three commonly used controls. The FMO control provides a measure of the spread of fluorescence (spillover) from the other fluorochromes into the channel of interest, and is recommended for accurately determination of the threshold for positive staining in multicolor experiments [432]. It does not, however, provide any measure of non-specific binding. In the phenotyping assays in **paper I and II**, isolated FMO controls for the activation markers were tested in initial experiments and compared with staining where several of the activation markers were omitted at the same time. In most cases the application of the negative gates was similar when excluding one or several of these fluorochromes and a "FMO minus more than one marker" was then used. The FoxP3 gates in **paper II** were based on biological negative populations.

A limitation with isotype controls is that the individual antibody conjugates have various levels of background staining, depending upon their specificity, concentration, fluorophore/protein ratio etc and they do not solve the problem with spillover from other channels [427, 432]. To account for these limitations in the phenotyping assays in **paper III**, all antibodies in the other channels were combined with fluorochrome/protein ratio concentration matched isotypes for the activation markers. Eventually, for the stimulation experiments mock-stimulated samples served as negative biological controls (see section 4.4.5).

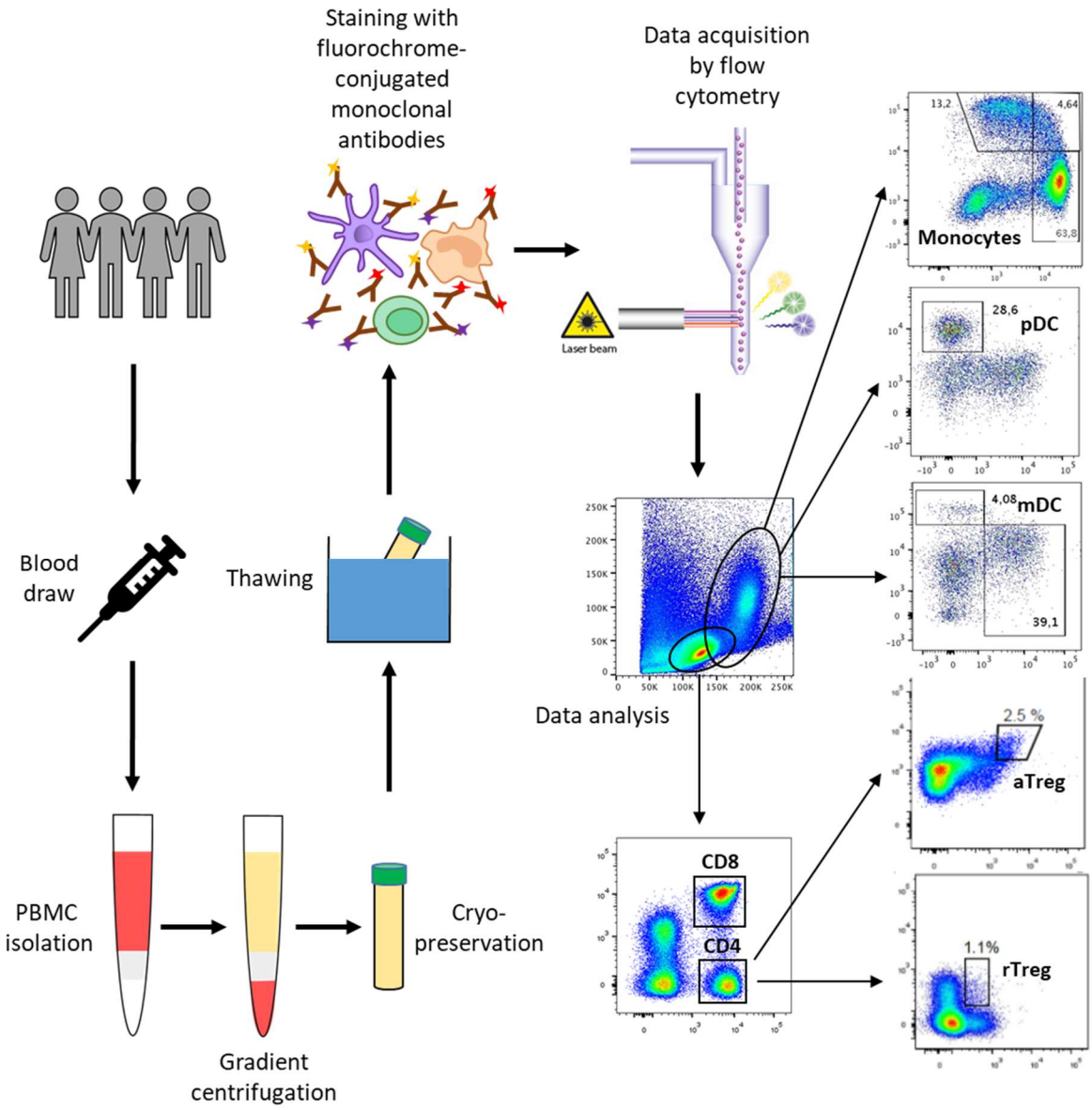


Figure 17. Logistics in flow cytometry experiments in paper I, II and III.

4.4.3 T cell phenotyping

After cryopreservation, an additional lymphocyte population at the FSC and SSC plot is often seen and it appears to be reduced during resting [410]. A strict lymphocyte gate was set since a higher proportion of dead cells are found within the left lymphocyte region [127, 410, 427]. However, this strategy might have excluded some lymphocytes [433], especially in **paper II** where the gates were most tight. More precisely we saw that the fraction of PD-1 positive $CD4^+$ and $CD8^+$ T cells were higher in this left population than in the lymphocyte population with higher FSC, which could have

affected the somewhat unexpected conclusion of similar PD-1 expression in INR and IR in **paper II**. Nevertheless, exclusion of dead cells is important in order to reduce the background staining [427] and re-gating with inclusion of this left population did not affect our results. In lack of available fluorescence channels for a live/dead marker, we therefore chose this gating strategy. Although the viability (calculated by Countess cell-counter) was lower in the flow cytometry experiments in **paper II**, we found no correlations between the viability percentage and the fractions of any of the analyzed cell populations.

In combination with anti-CD3, we used only anti-CD4 or anti-CD8 to define $CD4^+$ or $CD8^+$ T cells. Such a strategy raises the possibility for including double positive cells [433]. In **paper I**, a wider $CD3^+CD8^+$ gate was chosen to include $CD3^+CD8^+$ dim cells [433]. The approach with strict $CD4^+$ and $CD3^+$ gating in **paper II** was later checked against expanded gates. Larger gates gave an increase of ca 1% and 2-4% $CD4^+$ or $CD8^+$ T cells in the Tregs and activation assays, respectively. However, we observed only minor changes in the fractions of Treg subsets or activated or exhausted $CD4^+$ or $CD8^+$ T cells.

Based on the prognostic values of the expression of HLA-DR and CD38 (see section 1.5.4), T cell activation was characterized as HLA-DR and CD38 double positive $CD4^+$ or $CD8^+$ T cells in all three papers.

In addition to the surface markers CD45RA and CD45RO, the chemokine receptor CCR7 that mediates homing to lymph nodes, CD27 a member of the TNF receptor family, and the co-stimulatory molecule CD28 required for T cell activation and survival, are the most commonly used markers for phenotyping of T cell differentiation [427, 434, 435]. CD62L is degraded following cryopreservation and therefore less useful [406]. Due to limited number of channels in the flow cytometer, T cell differentiation was defined by staining with anti-CD45RA, anti-CD45RO and anti-CD27 as naïve ($CD45RA^+CD27^+$), central memory (CM) ($CD45RO^+CD27^+$) and EM ($CD45RO^+CD27^-$) $CD4^+$ or $CD8^+$ T cells. Some EM cells that lack CCR7 still express CD27 [436], which describes an intermediate memory subset often seen in untreated HIV infection [435]. It is therefore recommended to include CCR7 [434].

However, the correlation of naive T cells defined as $CD45RA^{+}CD27^{+}$ or $CD45RA^{+}CCR7^{+}$ is shown to be good [437] and the terminally differentiated effector cells are well defined by CD45RO and lack of CD27 [435]. PD-1 provides a negative co-stimulatory signal to T cells and their expression was used as a marker of T cell exhaustion [120].

4.4.3.1 Regulatory T cells (Tregs)

IL-2 and FoxP3 are essential for both differentiation and function of Tregs [438, 439] and Tregs are characterized by their expression of FoxP3 and high levels of CD25, as well as down-regulation of the IL-7 receptor expression [131]. Traditionally Tregs have been characterized in different ways as $CD4^{+}FoxP3^{+}CD25^{++}$, $CD4^{+}CD25^{++}CD127^{low}$ or $CD4^{+}FoxP3^{+}CD25^{++}CD127^{low}$. However, a recommendation proposed by an international workshop is now to define Tregs as $CD4^{+}FoxP3^{+}CD25^{++}CD127^{low}$ [440]. In **paper I** we used the surface markers CD25 and CD127, simplifying the lab-protocol by omitting the intracellular staining of the transcription factor FoxP3. Recent research has though shown that this approach can give an overestimation of percentages of Tregs compared to inclusion of FoxP3 [440]. Defining the limits of the CD25 and CD127 gate can also be challenging, and was in **paper I** done subjectively, but consistent between the two time-points. Recently, use of $CD3^{neg}$ or $CD3^{+}CD4^{neg}$ cell populations has been suggested to create a more objective gating strategy [440].

Furthermore, since not all of *ex vivo* isolated $FoxP3^{+}CD4^{+}$ T cells are regulatory and contain cytokine-secreting non-Tregs, the combination of CD25 and CD127 does not always isolate functionally pure Tregs [131, 441]. This highlights the rationale for studying different Treg subsets in disease. ATregs and rTregs are traditionally separated by their expression of CD45RA and FoxP3; aTregs; $CD45RA^{-}FoxP3^{high}$ and rTregs; $CD45RA^{+}FoxP3^{+}$ [131, 440, 441]. CD147 is also a marker of activated and highly suppressive human $CD45RO^{+}$ Tregs within the $CD4^{+}FoxP3^{+}$ subsets [442]. Characterization of aTregs with surface markers $CD25^{high}$ and $CD147^{high}$ is less demanding, allowing for functional live assays and has not been studied in HIV infection. We therefore included this cell population in addition to traditionally defined aTregs. Still, defining the boundaries of $CD25^{high}CD147^{high}$ cells can be difficult and we

looked at the FoxP3 expression when placing the gate as a way to make a more consistent gating strategy.

The percentages of aTregs were in line with previous reports showing 1-4 % aTregs of $CD4^+$ T cells in healthy adults, whereas the levels of rTregs were low [440, 441]. Both the cryopreservation, the rapid dilution during thawing and lack of resting could have contributed to the relatively low percentages of Tregs [405, 410], although other studies found similar frequencies of Tregs in both fresh and cryopreserved samples with different thawing procedures [443, 444]. However, Treg subsets constitutes a small proportion of the $CD4^+$ T cells increasing the risk of error. Ideally, optimizing protocols with fresh samples containing higher number of live lymphocytes would have been preferable.

4.4.3.2 Th17 and Tc17 cells

Based on Th17 cells important role for maintaining gut immunity, they were studied in peripheral blood in **paper I**. They are non-naïve $CD4^+$ T cells and defined by their production of IL-17 [445]. They originate from naïve thymic Th17 precursors expressing the surface marker CD161 and the transcription factor RORC [445-447]. All mature Th17 cells also express CD161, but not all CD161 positive cells produce IL-17 and non-classic Th1 cells developed from Th17 are also shown to express CD161 [446, 447]. Furthermore, Th17 cells can be described by presence of surface molecules CCR6 and absence of CXCR3. The phenotype assay in **paper I** included exclusively surface markers. Our characterization of the Th17 cells as $CD4^+CD45RA^-CD161^+$ was thus not as precise as when measuring intracellular IL-17 production, but should include these cells.

IL-17 is also produced from a population of $CD8^+$ T cells; Tc17 cells/ MAIT cells and appear to be contained within the $CD8^+CD161^{++}$ cell population where RORC also is highly expressed [448]. We therefore defined Tc17 cells as $CD8^+CD161^{++}$ and the frequency of these cells in **paper I** was in line with other reports from HIV-infected [449, 450].

Table IV. Markers used in flow cytometry assays.

Marker	Function	Expressed	Marker of	Ref.
CD3	T cell signal transduction	T cells	T cells	[451]
CD4	Initiates the early phase of T cell activation, binds MHC II	T cells, Mφ, monocytes, granulocytes	T helper cells	[452]
CD8	T cell mediated killing, binds MHC I	T and NK cells	T cytotoxic cells	[452]
HLA-DR	MHC II surface receptor, binds peptides derived from endocytosed proteins, antigen presentation	Highly expressed on monocytes, Mφ and DC, upregulated on activated T, B and NK cells	Monocytes, DC, activation	[453]
CD38	Cell adhesion, signal transduction, lymphocyte differentiation	T, B cells and NK cells, DC, Mφ, monocytes, stem cells	Activation	[454]
PD-1	Negative regulator of activated T cells	T and B cells	T cell exhaustion	[120]
CD161	Associated with memory phenotype and IL-17 production, co-stimulator for TCR, marker for pro-infl. Tregs	T and NK cells	Th17 and Tc17 cells	[445, 448, 455, 456]
CD45RA	CD45 isoform	T, B and NK cells, DC, Mφ, monocytes, stem cells	Naïve T cells	[457]
CD45R0	CD45 isoform	Hematopoietic cells	Memory T cells	[457]
CD25	Receptor for IL-2, promotes IL-2 responses	T, B and, NK cells, Mφ, monocytes	Tregs	[458]
CD127	Receptor for IL-7, regulating naïve and memory T cell homeostasis, low expression in Tregs	T cells, monocytes, Mφ, stem cells	Tregs	[459, 460]
FoxP3	Forkhead family transcription factor, expressed by and required for development of Tregs	T cells	Tregs	[131]
CD147	Induction of matrix metalloproteinases, T cell receptor signaling, marker of T cell activation and suppressive function of Tregs	T, B and NK cells, Mφ, monocytes, granulocytes	Activated Tregs	[461]
CD45	Regulator of T- and B cell antigen receptor signaling, cell growth and differentiation	Hematopoietic cells	Hemato-poietic cells	[462]
CD14	LPS receptor, mediates innate immune responses to LPS	Monocytes, Mφ, granulocytes	Monocytes	[463, 464]
CD16	Binds Fc region of IgG, mediates phagocytosis and antibody-dependent cellular cytotoxicity	Monocytes, Mφ, DC, T and NK cells, granulocytes	Monocytes	[465]
CD11b	Endothelial adhesion for monocytes, Mφ and granulocytes	T, B and, NK cells, monocytes, Mφ, DC, granulocytes	Activation	[466]

Table IV. Cont.

Marker	Function	Expressed	Marker of	Ref.
CD163	Clearance and endocytosis of hemoglobin/haptoglobin complexes	Monocytes, Mφ,	Activation	[266]
CD142	Tissue Factor, initiates extrinsic blood coagulation cascade	Macrophages, Mφ,	Activation	[246, 262]
CD1c	BDCA-1, antigen presentation	DC, T and B cells, Mφ, monocytes	mDC	[234, 467]
CD141	BDCA-3, thrombomodulin, antigen cross-presentation	DC, monocytes, Mφ, granulocytes	mDC	[468]
CD303	BDCA-2, antigen capture, cell adhesion	DC	pDC	[234, 469]
CD86	Binds CD28, co-stimulator for T cell activation. Binds to CTLA-4 and negatively regulates T cell activation	DC, monocytes, Mφ, B and T cells	Activation	[470, 471]
IP-10	CXCL-10, CXCR3 ligand, migration of cells to inflamed tissue and lymph nodes, stimulates immune activation, mediates apoptosis, cell growth inhibition, attenuate angiogenesis	Monocytes, DC, T, B and NK cells	Activation	[270, 278, 472]
IDO	Rate-limiting enzyme for catabolism of tryptophan through the kynurenine pathway	Monocytes, Mφ, DC	Activation	[473]

4.4.4 Phenotyping of monocytes and dendritic cells

4.4.4.1 Monocytes

To avoid activation of the monocytes from polystyrene, sterile polypropylene tubes and plates were used for all flow cytometry experiments in **paper III** [413].

According to recommended nomenclature in the literature, the different monocytes subsets were defined based on their CD14 and CD16 expression in classical: $CD14^{++}CD16^{-}$, intermediate: $CD14^{++}CD16^{+}$ and non-classical: $CD14^{+}CD16^{++}$ monocytes [234]. Following the findings of upregulation of monocyte activation markers in PLWH on ART [240, 243, 246], we chose to analyze TF (CD142), CD163, CD11b and HLA-DR. CD11b is from the β2 integrin family, involved in monocyte adhesion and endothelial transmigration. CD11b is constitutively expressed and increases after pro-inflammatory stimuli [466]. Monocytes express high levels of HLA-DR (MHC II) [434] that reflects activation status of the monocytes. In line with other studies CD11b was highly expressed [243, 413], almost all classic and

intermediate monocytes were positive for CD11b and retrospectively another activation marker as for instance CD40 or CD86 could have been more interesting to analyze.

NK cells and lymphocytes can also express CD16 and should be excluded to avoid contamination with these cells [434]. Thus, monocytes were gated as HLA-DR$^+$ and CD3, CD19, CD20 and CD56 negative cells and dead cells were also excluded. A rectangular gating strategy of the monocyte subsets has been recommended which we performed [474].

Monocytes are typically distributed in approximately 85% classical monocytes and the remaining in intermediate and non-classical [238]. The fraction of classical monocytes was median 87.2% (IQR, 81.2-91.4), the non-classical median 10.1% (6.1-15.9), while the proportions of intermediate monocytes were lower in all our cohorts with a median of 2.4% (1.6-3.5) compared with approximately 5% in other studies of PLWH on ART using cryopreserved PBMC and almost similar gating strategy without FMO [245, 475]. We attempted to standardize the application of the gate between classic and intermediate monocytes, however, use of FMO or isotype for CD16 would have been preferable and could possibly have given higher proportion of intermediate monocytes.

The basal *ex vivo* CD142 expression was low, but in line with other studies [244, 246]. However, in initial experiments with the same antibody we observed a profound increase in CD142 after four hours stimulation with LPS, indicating that the antibody was functioning.

4.4.4.2 Dendritic cells

The number of DCs in the blood is low, and monocyte-derived DCs from culturing monocytes, are therefore often used for functional assessments. However, mDCs and pDCs arise from another precursor [476] and monocyte-derived DCs seems to be a separate functional group of antigen presenting DCs and do not necessarily represents the phenotypical characteristics and functions of mDCs, and in particular not pDCs [233, 477]. Despite the challenges with small cell populations, phenotyping of the circulating DCs probably reflects the *in vivo* situation better.

As the commonly used markers CD11c and CD123 are not DCs specific and expressed by several lymphoid and myeloid cells [234, 413], CD1c and CD141 are currently recommended markers for defining the two subtypes of mDCs and CD303 for pDCs [234, 476]. A good concordance between $CD303^+$ and $CD123^+$ pDCs has also been shown [413]. Similar to monocytes, gating of DCs is based on selecting HLA-DR expressing cells and exclusion of dead cells, B cells, T cells and also monocytes [413, 478]. CD56 and CD16 were not included as NK cells do not express CD1c, CD141 or CD303, and CD1c and CD141 positive mDCs have been shown to be CD16 negative [478].

Upon exogenous stimuli including exposure to HIV, DCs mature and upregulate several activation markers (CD80, CD86, CD40, MHC II). Thus, we analyzed the expression of CD86, the co-stimulatory molecule for T cells, HLA-DR (MHC II) and the maturation marker CD83 [204, 477, 479].

4.4.5 Intracellular assays and stimulation with inactivated HIV

Based on the findings of an inverse association between IDO activity and IP-10 levels with future CD4 count in **paper II**, we aimed to explore potential differences in IDO and IP-10 activity on a cellular level in INR and IR. Following HIV-stimulation *in vitro* DCs upregulate IDO [66, 189, 190], and IP-10 has been shown to be secreted mainly from monocytes and DCs [278], giving rationale for studying the IP-10 and IDO responses in these cell types. We evaluated the effect of HIV-exposure upon IDO and IP-10 upregulation in the different cohorts. Ideally, inclusion of several different stimuli such as LPS, unmethylated CpG-containing DNA and for instance CMV, could have given a more comprehensive knowledge of factors contributing to monocyte and DC activation in INR [67, 151, 196]. However, with limited amount of PBMC this was not possible.

Previous experiments have shown that productive infection is not necessary for HIV-induced IDO or IP-10 responses [189, 278]. Thus, we chose to use AT-2 HIV with microvesicles (mock) as negative control for our experiments. These reagents were provided by the AIDS and Cancer Virus Program, Leidos Biomedical Research,

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AT-2 inactivation covalently modifies thiol groups in internal viral proteins including the nucleocapsid protein that is required for infectivity, while the envelope glycoproteins remain intact. This manipulation restricts HIV-replication before reverse transcription, but does not affect the binding and viral uptake in target cells [480]. Non-viral particles, so-called microvesicles (mock) can co-purify with virus. They bud from the cell membrane and contain various proteins including HLA-DR and β2M, and can therefore act as cellular antigens [481]. In order to eliminate the component from nonspecific stimulation, mock isolated from uninfected cell cultures and with equivalent protein concentration is recommended as negative controls. AT-2 HIV responses were calculated with subtraction of the corresponding mock stimulated control. Due to a limited number of cells, we did not include an unstimulated control that also could have given valuable information about the constitutive IP-10 and IDO expression in monocytes and DCs in the different study cohorts. Nevertheless, the initial experiments showed almost similar IP-10 and IDO expression in unstimulated and mock stimulated samples.

We used dual-tropic, CXCR4 and CCR5 AT-2 HIV as previous studies found similar activation of monocytes and DCs as well as IDO upregulation with CXCR4 or CCR5 tropic viruses [189, 482]. Furthermore, other studies evaluating IDO or IP-10 responses in monocytes or DCs after AT-2 HIV stimulation have used AT-2 HIV concentration of 400-500ng/ml p24 and culturing time between 20 and 48 hours [189, 278]. Based on initial experiments stimulating with either 500 or 1000 ng/ml p24 dual tropic virus for 8, 18 or 24 hours culturing time, we concluded with best results and favorable logistics when stimulating with AT-2 HIV at a concentration of 500ng/ml p24 for 18 hours.

With a secretion inhibitor, secreted cytokines and proteins can be retained intracellularly. Monensin is more toxic and already after 6 hours treatment monocytes are less viable than when using brefeldin A [483]. Brefeldin A was therefore chosen and added after six hours to allow for intracellular processing of the AT-2 HIV as well as

leakage of secreted cytokines from pDCs in particular, which has shown to be important for the activation of both monocytes and mDCs after HIV-stimulation. This time-point was in line with previous studies analyzing IP-10 expression in, or activation of monocytes and DCs [278, 482]

Unfortunately, the CD303 expression disappeared in the HIV-culture assay, probably due to downregulation and internalization following stimulation [484, 485], and we were therefore unable to evaluate the IP-10 and IDO responses in pDCs. Nevertheless, IFNα2 is mainly secreted from pDCs, and reflects pDCs response [221].

Inclusion of a positive control is recommended in stimulation experiments as a test of correct performance of the experiments if results in the other samples come out negative [432]. In the test assays we detected relatively robust IDO and IP-10 responses after HIV-stimulation and in the choice of a mock-stimulated or a positive control, due to limited amount of PBMC, we concluded with use of mock.

Finally, exploring *in vitro* responses in different DC subsets is challenging since they constitute rare cell populations and sometimes there were too few cells to evaluate IP-10 and IDO responses. However, studying PBMC have an advantage over isolated cell-populations as cytokine responses and in particular IP-10, are dependent of cross-talk between different immune cells [486]. Enrichment of pDCs and mDCs in PBMC could have been an option if we have had more patient samples.

4.5 Microbiota analyses

High through-put sequencing of marker genes in gut microbiota yields important information on both microbial diversity and composition and is a widely used technology. It is cheaper and easier to conduct than full metagenome sequencing, however, microorganisms can have different number of copies of the targeted gene giving more inaccurate results and it does not detect all low-abundant taxa [487]. Furthermore, multiple parameters during the analyze process can influence the results making comparisons between studies challenging [488].

All microbiota samples were handled using the same protocol. The DNA yield and microbiota composition can be affected by methods of extraction [487]. The fecal samples were thawed on ice and kept cold during homogenization and bacterial cell lysis to avoid DNA degradation. DNA was extracted from supernatant lysate solution using a DNA extraction kit, followed by PCR amplification targeting the 16S ribosomal RNA (rRNA) gene, which is ubiquitous in procaryotes. The 16S rRNA gene consists of nine variable regions that are flanked by highly conserved DNA, making it ideal for universal primer sites [487, 488]. The choice of primers and subsequently hypervariable regions analyzed will influence the sequence dataset as regions of variability may sometimes be biased toward certain species [489, 490]. Universal primers flanking the commonly utilized V3-V4 region of the 16S gene modified with addition of TruSeq Illumina adapters were used in this project. PCR amplification consisted of 30 cycles followed by a final elongation. Sequencing was performed at the Norwegian Sequencing Centre on the Illumina MiSeq platform using the 300 bp paired-end kit.

The next step is to convert the 16s rRNA gene amplicons into microbial data using a bioinformatics pipeline. Reads are clustered into operational taxonomic units (OTUs), which are grouped on the basis of DNA sequence similarity >97%, and then classified by using a reference database [487]. The quality of the interpretation of these data is dependent of the quality of the sequencing process. Thus, it is important to check all sequencing reads and to remove low-quality reads and also chimeras, which are artifacts of PCR amplification [488]. Reads were sorted per sample based on unique forward and reverse barcode combinations, quality controlled and then mapped using default values in "closed reference OTU clustering" in QIIME 1.8.0 [491] against the Greengenes database version 1308 [492]. OTUs containing less than two reads were filtered out, and the resulting OTU table was subsampled to 9442 reads per sample. Taxa summary plots, alpha diversity and beta diversity analyses were based on this OTU table, using QIIME software.

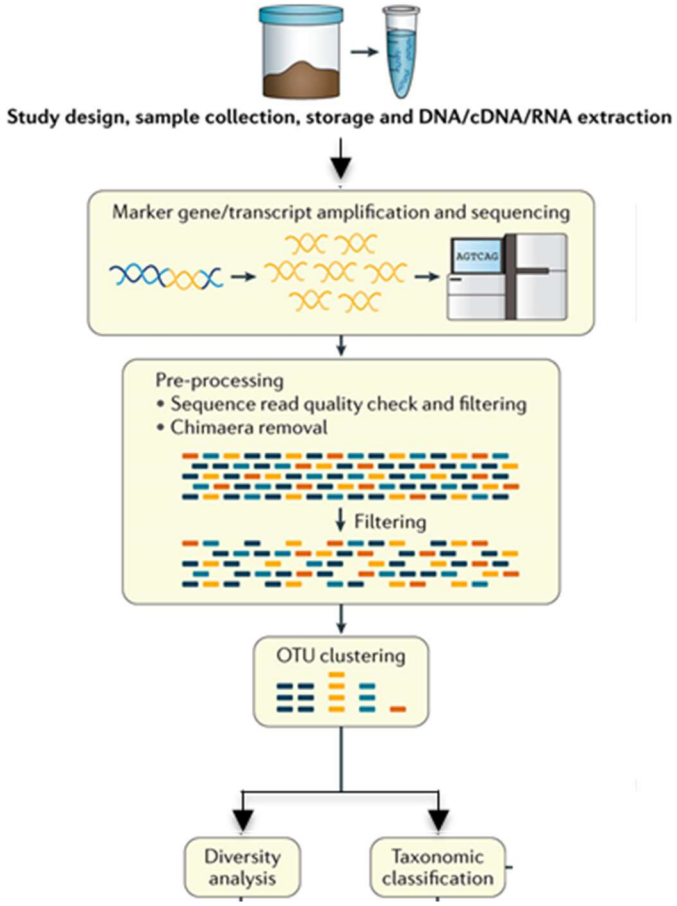

Figure 18. Flowchart of the major steps involved in 16S rRNA sequencing of the fecal samples.

Modified by permission from Springer Nature: Nature Reviews Gastroenterology & Hepatology. A clinician's guide to microbiome analysis. Marcus J. Claesson, Adam G. Clooney, Paul W. O'Toole, 2017. (License number: 4592641011286)

Several environmental factors greatly affect the composition of the gut microbiota, such as BMI [493], dietary patterns [494, 495], use of antibiotics [488, 496, 497] and sexual preferences [176]. The study groups had no significant differences in the fraction of MSM, comorbidities or waist circumference. 53% were MSM. They were asked to maintain their diet throughout the study period, but we did not record any diet questionnaire. We cannot exclude differences in diet as a potential confounder contributing to the tendency of higher abundance of Bacteroidetes and *Bacteroides* in those randomized to probiotics at baseline, although possible dietary changes during the study period are less likely to have influenced the results of the microbiota analyses [495]. Use of antibiotics or probiotics the last two months were exclusion criteria. One study has shown that the taxonomic composition was mainly restored to the pretreatment

state four weeks after ciprofloxacin use, but some taxa failed to recover within six months [497], and therefore we cannot rule out an antibiotic effect on the microbiota composition. Furthermore, one participant in the probiotic group had a short course of per oral penicillin in the first half of the intervention period. However, this person had changes in the analyzed phyla and genera from baseline to week eight that were lower than median for the probiotic group, except for an increase in *Bifidobacterium* and Actinobacteria above the median values.

4.6 Statistical considerations

As a consequence of relatively small sample sizes combined with biological data that often are skewed, there was generally a lack of conformation of data to a normal distribution. Non-parametric statistics were therefore applied in all papers. Non-parametric statistics are based on fewer assumptions and the observed values are ordinal ranked making them usually more robust against extreme variables. At a cost, non-parametric test may be less powerful for detecting differences [498]. We used Wilcoxon Test for paired samples and Mann Whitney U Test when analyzing only two groups. For comparison between more than two groups Kruskal-Wallis Test followed by Mann Whitney U Test in **paper II,** and followed by Dunn's posthoc test with correction for multiple comparisons in **paper III**, were applied. Differences in categorical variables were assessed by Fischer's Exact Test or Pearson Chi square test and Spearman's rank correlation for calculation of correlation between parameters. Markers with levels significantly different between the main cohorts in **paper II**, and factors associated with prospective CD4 gain in **paper III** were further adjusted for age, nadir CD4 and duration of viral suppression or ART by binary logistic regression. Age and nadir CD4 were selected since these factors have been shown to be main predictors of CD4 recovery (see section 1.6.3.1). Furthermore, as the CD4 count is known to gradually increase after ART start, duration of viral suppression or ART were therefore included in the model (see section 1.6.2). However, adjustment for several predictors in small sample sizes as in **paper III** in particular, results in large confidence intervals and

the results should therefore be interpreted with some caution. We used a two-tailed significance level of 0.05.

A main statistical concern in these studies was the relatively low number of study participants, increasing the risk of statistical type II errors and failure to detect a true effect of intervention or true differences between groups [499]. *I.e*, a false null hypothesis is not rejected. Small samples may not either be representative for the entire population. Power calculations can be used to calculate the number of needed participants to detect a given difference with a given probability. Nevertheless, in experimental research it is difficult to predict the size and variations of the true difference that are needed for power calculations. Considerations about sample size calculations in **paper I** are discussed in section 4.1.2.

Performing numerous statistical analyses without correction for multiple comparisons give higher probability for detecting effects or differences that are not present, *i.e*. type I statistical errors and incorrect rejection of the null hypothesis. However, in exploratory trials is important not to discard potential relevant observations, and p-values are rather hypothesis supporting and not interpreted as confirmatory. Adjustments for multiple testing were therefore not performed in **paper I and II**, but in **paper III** we chose to use Dunn's posthoc, pairwaise, multiple comparisons procedure being aware that such correction potentially could increase type II errors.

In randomized controlled trials the "intention to treat" analysis is preferred to preserve the benefit of randomization [500]. This was hard to achieve in **paper I** because of loss to follow-up and thereby missing outcomes. Thus, as-treated analyses were limited to subjects who had available data both from baseline and week eight. Ideally the three different randomized groups should have been analyzed separately. However, it was outlined in the study protocol that if no differences were observed between placebo and controls, the probiotic study arm would be compared with the placebo and control arm combined.

In **paper III**, to reduce the %CV in the flow cytometry experiments, small cell populations were excluded for analyses of activation markers [501, 502]. This was done

prior to statistical calculations. We cannot rule out that excluded cell populations could have displayed special characteristics as for instance being more activated, since the healthy controls had adequate cell population sizes. However, the exclusions were few, largely from the $CD141^+$ mDC population, and equally distributed between the HIV-positive study-groups, except for the HIV-stimulation assay where the excluded $CD141^+$ mDC populations for analyses of percentages positive for IP-10 were mainly from the IR group.

5. Discussion

5.1 Does probiotic intervention reduce microbial translocation and systemic inflammation in PLWH on ART with subnormal CD4 count?

Considering the presence of gut microbial dysbiosis in PLWH that potentially contributes to microbial translocation and increased inflammation and immune activation (see section 1.5.7 and 1.5.8), there has been a major interest in different approaches attempting to modulate the gut microbial composition in a favorable direction and thereby reducing inflammation and improving prognosis for these patients. Probiotics are "living bacteria or fungi that confer a health benefit for the host" and typically consists of multiple strains of bacteria (e.g *Lactobacillus spp.* and *Bifidobacteria spp.*) [503]. The effects of probiotic are mainly strain specific [503]. When this study (**paper I**) was initiated there were no reports exploring the potential role of probiotics in reducing microbial translocation in PLWH on ART. Later on, the possible association between *Lactobacilliales* and CD4 levels, reduced microbial translocation and immune activation even in ART-treated [171, 180] as well as the reported beneficial effect of *Lactobacillus* on IDO activity [192], have underscored the rationale for exploring the effect of supplementation with these bacteria.

Eight weeks of probiotic supplement did not have any impact on the primary endpoints in our pilot study (**paper I**). In line with other recently published probiotic intervention studies on PLWH on ART, no serious adverse events or significant changes in CD4 counts were recorded [348, 504-506]. When the study was initiated, patients with severe pancreatitis supplemented with probiotics containing different strains of *Lactobacilli* and *Bifidiobacteria*, were shown to have increased mortality [507], addressing the importance of evaluating safety in PLWH. Moreover, no changes in the primary readouts LPS and sCD14 as markers of microbial translocation or T cell activation were detected. As CRP, IL-6 and D-dimer were increasingly reported as important prognostic

markers in ART-treated in the period after the study was outlined, we focused on these markers.

Following the intervention period the participants randomized to probiotics (**paper I**) showed reduced levels of D-dimer and trends towards lower levels of CRP and IL-6, although there were no significant differences in the inter-individual changes between the probiotic and non-probiotic groups. Moreover, in those randomized to probiotics we observed significant alterations in the stool composition with increased relative abundance of the Actinobacteria and Firmicutes phyla, *Bificobacterium* and *Lactobacillus* genera and Ruminococcacea family, while the fraction of Bacteriodetes and *Bacteroides* were reduced. Since then, several intervention studies with probiotics, prebiotics and synbiotics have been undertaken. Prebiotics are non-digestible compounds such as oligosaccharides that can stimulate growth of beneficial gut bacteria [503] and synbiotics refers to a combination of both probiotics and prebiotics. However, most of these intervention studies have been small, using many different probiotic strains or various kinds of prebiotics in diverse HIV-cohorts, making overall conclusions difficult. Furthermore, information about use of antibiotics or diet have often been sparse or lacking [165, 508].

Villar-Garcia *et al* also included patients with sub-optimal CD4 recovery on ART and found significant reduced LPS-binding protein (LBP), IL-6 and a tendency towards reduced hs-CRP after 12 weeks of intervention with the probiotic yeast *Saccharomyces boulardii* compared with placebo [504]. Only minor changes were detected in the stool [509]. In a pilot study with patients on stable ART for more than three years and adequate immune recovery, probiotic supplementation containing nine different bacterial strains (Visbiome) including *Lactobacillius acidophilus* for 48 weeks reduced both hs-CRP, IL-6 and T cell activation (HLA-DR^{+}CD38^{+}) to similar levels as HIV-negative controls [505]. However, fecal samples were not collected, no control or placebo group were included, and several background factors such as comorbidities and use of antibiotics were lacking. Thus, the observed reduction in inflammation parameters could potentially be biased by other factors. Results from the largest study investigating the effect of a multistrain probiotic (Visbiome ES) to ART-treated were

presented at CROI in 2019 (NCT02706717). Forty-seven received probiotics and 46 placebo for 24 weeks although only 73 were analyzed per-protocol. The relative abundance of Gammaproteobacteria was reduced and there was a trend towards increased proportions of *Lactobacillus* and *Bifidobacterium* [506]. Still, they observed no changes in markers assessing microbial translocation, inflammation, monocyte- or T cell activation [506]. However, the included patients were well-treated with a median CD4 count at 712 cells/μL, and adherence was a reported challenge in the sub-study presented in 2018 [510]. Another pilot study from our research group exploring the impact of four probiotic strains including *Lactobacillus rhamnosus* and *acidophilus* for eight weeks administered to 20 immunological non-responders discovered trends towards reduced levels of enterocyte damage (I-FABP), microbial translocation (sCD14 and LBP) and IL-22 producing $CD4^+$ T cells in lamina propria (NCT02640625) [348]. The relative abundance of Bifidobacteria increased combined with an expansion of α-diversity. In contrast, *Lactobacillus rhamnosus* supplement for eight weeks to virally suppressed well-treated or viremic untreated showed no changes in plasma inflammation markers although intestinal inflammation assessed by PET/MRI was reduced [511].

Intervention studies with prebiotics have been largely undertaken in viremic PLWH. Supplement with prebiotics containing oligosaccharides and glutamine over six weeks has been reported to attenuate HIV-associated dysbiosis in viremic PLWH with an increase in Firmicutes, Actinobacteria and butyrate-producing bacteria, whereas no such changes were observed in ART-treated including INR patients [177]. A reduction in T cell activation was seen in all PLWH, but there were no changes in D-dimer or soluble inflammation markers. Two large studies investigating the effect of one year intervention to viremic PLWH with two different immunomodulatory nutritional products including oligosaccharides and other immunonutritients compared with placebo, showed inconsistent results. Serrano-Villar *et al* detected no differences in CD4 counts, CD4/CD8 ratio, T cell activation, microbial translocation, inflammation or diversity in viremic with advanced HIV-disease [512]. In patients with a somewhat shorter interval since HIV-diagnosis and higher CD4 count, the intervention group

experienced a lower $CD4^+$ T cell loss [513]. However, both these studies had adherence issues and a relatively high drop-out rate [512, 513].

With the positive effect of fecal microbial transplantation (FMT) to *Clostridium difficile* colitis in mind, a pilot study of FMT to ART-treated with confirmed gut dysbiosis was conducted. Eight weeks later the recipients showed only partial engraftment of the donor microbiome and no changes in systemic inflammation parameters were detected [514].

Experiments trying to block the microbial translocation in PLWH on ART have been mainly disappointing. Neither long-term intervention with hyperimmune bovine colostrum that binds luminal LPS, nor four weeks supplement with rifaximin, a non-absorbable antibiotic that decreases LPS in cirrhotic patients, showed any effect on CD4 count, or markers of microbial translocation and inflammation at end of treatment in INR patients [377, 515]. A possible beneficial effect on sCD14, IL-6 and CRP was seen four weeks post-treatment in rifaximin treated [515]. Administration of oral recombinant lactoferrin, an iron-binding protein that binds LPS, did not induce any improvement in inflammation markers, monocyte- or T cell activation, mucosal integrity, gut microbial diversity or composition in PLWH on ART with a normal CD4 gain [516]. Recently, results from an intervention in well-treated PLWH with retinoic acid that are important for maintenance of IL-17 and IL-22 producing cells in the gut, were presented. Surprisingly, cellular and soluble markers of inflammation increased after 16 weeks compared to placebo, but were not sustained three months after removal of therapy. However, the intervention group experienced significantly increased CD4 count at this time-point [517]. In a single-arm study of viremic PLWH with high CD4 count, a transitory effect on soluble TF and cholesterol levels was seen after eight weeks supplement with the phosphate-lowering drug sevelamer. Interestingly, D-dimer increased, but there were no other changes in inflammation or microbial translocation parameters [518].

In conclusion, the attempts to modulate the gut microbial composition have shown limited effect upon microbial translocation and inflammation in HIV infection. However, administration of probiotics to PLWH with sub-optimal CD4 recovery on ART seems to have some beneficial impact on soluble inflammation markers, as

reported by our study (**paper I**) and others [348, 504]. As low nadir CD4 counts predict gut dysbiosis and enterocyte damage in PLWH [172], together with indications of a more disturbed gut immunity (see section 1.6.3.1), probiotic or other microbiota-directed interventions, to this group of patients could be of potential benefit. Zmora *et al* showed that individuals with significantly lower baseline levels of the probiotic strains in the lower gastrointestinal mucosa were more permissive to colonization with the probiotics administered [519].

Despite reduced levels of inflammation markers in our study, we did not detect any improvement in LPS or sCD14 as markers of microbial translocation. Most of the abovementioned intervention studies report similar results except for Villar-Garcia *et al* and Meyer-Myklestad *et al* [348, 504]. Of note, no studies have observed reduction in levels of LPS [377, 511, 515]. LPS' pro-inflammatory capacity is dependent of the degree of acetylation [520]. To investigate this further we have found that the abundance of gut bacteria associated with pro-inflammatory hexa-acylated LPS was very sparse compared with the presence of bacteria associated with non-inflammatory penta-acylated LPS [521]. The individuals with the highest ratio of hexa-acylated LPS to penta-acylated LPS producing bacteria had increased levels of neopterin and KTR. After the probiotic intervention, there was a decrease of Gram-negative bacteria which correlated with the changes in plasma LPS levels. This reduction was seen in bacteria related to penta-acylated LPS production and in not Gram-negative bacteria related to hexa-acylated LPS [521]. Thus, plasma LPS seems to mainly reflect LPS with much lower TLR4 stimulatory potential and might explain why plasma LPS' correlation with other markers of inflammation is variable.

We observed changes in the stool consistent with the probiotic strains administered. Yet, interpreted with caution as the 16S rRNA analyses could not distinguish between the supplemented strains and endogenous species. Similar changes have been observed to varying degrees in other studies administering probiotics [348, 506, 511], but can be affected by the analyzing platforms [519]. Nevertheless, even if Zmora *et al* detected all the supplemented probiotic strains in stool samples, they showed that degree of the mucosal colonization was highly diverging between both different regions in the

GI-tractus and among the individuals [519]. Moreover, the stool composition did not reflect the degree of mucosal colonization, while the cecum distinguished best between colonization statuses. Dillon *et al* demonstrated that fecal intestinal aspirates and stool samples reflected the same altered patterns of Bacteroidetes families and genera, but the HIV-related changes in Proteobacteria and Firmicutes seemed to be mucosa specific [166]. Although we detected changes in the stool composition, these did not correlate with the reduction of the inflammation markers. Thus, future studies should not solely rely on stool samples as a correlate for intestinal probiotics colonization.

The effect of the probiotics can be attributed to taking up space and limiting growth of other microbes, stimulation of butyrate production, and enhancement of mucosal barrier integrity [503]. Probiotics induced transcriptional changes in several genes in the terminal ileum related to the immune system in the study by Zmora *et al* [519]. Gut mucosal biopsies would therefore have been valuable in elucidating potential mechanisms that could explain the observed systemic responses.

As discussed under methodological considerations, there are several methodological limitations in our study (**paper I**), in particular the low number of participants. Furthermore, in order to improve control of biasing factors, validated dietary questionnaires should be included in future studies.

5.2 Increased T cell activation and differentiation in INR

We aimed to further explore immune mechanisms and candidate markers for suboptimal CD4 recovery in response to ART. Considering low nadir CD4 count and higher age as the most well-known predictive factors for incomplete immune recovery, we selected INR and IR subgroups with similar age and nadir CD4 count for in depth analyses of T cell phenotypes in **paper II**. The INR patients displayed increased fractions of activated (HLA-DR$^+$CD38$^+$) and more differentiated CD4$^+$ and CD8$^+$ T cells with lower proportions of naïve (CD45RA$^+$CD27$^+$) and concomitantly higher fractions of EM T cells (CD45RO$^+$CD27$^-$). Our findings are in line with the literature showing evidence of higher levels of in particular activated CD4$^+$ T cells, but also activated CD8$^+$ T cells

[320, 323, 334, 522], even after adjustments for relevant factors [139, 325, 344]. Lower fractions of naïve T cells, especially $CD4^+$ T cells, is another hallmark of INR [320, 321, 334, 344, 523], and seem to be more often reported than increased T cell maturation [334, 524]. These results are consistent across different definitions of INR status.

The activated $CD4^+$ T cell phenotype in INR is accompanied by increased cycling of $CD4^+$ T cells [324, 325, 334]. Furthermore, chronic immune activation has been associated with T cell exhaustion, but in contrast to other reports [320, 321, 344, 347] we did not detect differences in the PD-1 expression in $CD4^+$ T cells between INR and IR. This result should be interpreted with caution as it could have been affected by a somewhat lower viability of the cells, or the strict lymphocyte gating strategy applied (see section 4.4.3). In addition, both T cell exhaustion and activation are linked to apoptosis [120, 122, 127, 323], and these connections turn out to be highly relevant in INR. In both previous studies [323, 324, 344] and recent research using supervised learning assessing the relevance of a large number of immune parameters [320, 321], $CD4^+$ T cell activation, exhaustion and death seem to be the strongest immunological predictors for INR status.

5.2.1 Skewed Treg subsets in INR

In spite of increasing evidence for a negative role of Tregs in immune recovery [138, 143, 324, 355, 525], few studies have investigated Treg subsets in INR. As different Treg phenotypes possess various suppressive capacities, analyzing these subsets adds important value [131]. **Paper II** presents novel data on the $CD4^+CD25^{high}CD147^{high}$ aTregs in relation to $CD4^+CD45RA^+FoxP3^+$ rTregs in INR. We found an increased aTregs/rTregs ratio with lower fractions of rTregs in INR compared with IR, and the highest aTregs proportion was seen in those with the lowest CD4 counts. This is in line with the sparse literature reporting higher aTregs percentages defined by $CD4^+CD25^{+/high}Foxp3^{+/high}CD45RA^-$, in virally suppressed PLWH with incomplete CD4 gain [139, 355]. In fact, fractions of aTregs (various definitions) came out as an important predictor for INR status when analyzing multiple immune parameters in the study by Perez-Santiago *et al* [321]. Nobrega *et al* found persisting low proportions of naïve Tregs and high proportions of proliferating $Ki67^+$ Tregs in PLWH starting ART

with CD4 count < 200 cells/µL [136]. In contrast, we show that the aTregs/rTregs ratio was increased in INR compared to IR independent of nadir CD4, in line with Gaardbo *et al* [139].

Impaired thymic output and accelerated proliferation to aTregs due to residual immune activation are possible explanations of the observed lower fractions of rTregs in INR [136, 146]. Whether these alterations in the Tregs subsets in INR are potentially unfavorable and responsible for the suboptimal CD4 recovery remains to be answered. We could not detect any relation to the prospective CD4 count, although the aTregs frequencies and aTregs/rTregs ratio were linked to levels of inflammation markers, in particular IP-10 and KTR, as well as higher levels of activation of both pDCs and mDCs (**paper III**). Furthermore, there was a negative association between the fractions of aTregs and HIV-specific monocyte-, DC- and cytokine responses, which was not seen in IR, indicating that aTregs could suppress innate and Th1 immune responses in INR. Previously, aTregs ($CD4^+CD45RA^-FoxP3^{high}$) have been associated with reduced HIV-specific $CD8^+$ T cell responses in PLWH [148]. Suppression of HIV-induced IDO and IP-10 expression in monocytes and mDCs could theoretically be beneficial, while lower levels of IFNγ may indicate lower T effector functions. Altogether, our results suggest that a high aTreg/rTreg ratio could play a role in impaired CD4 recovery and immune function in INR patients. However, taking the advantage of $CD4^+CD25^{high}CD147^{high}$ aTregs that allow for functional *in vitro* assays in presence or absence of these aTregs, studying T cell and innate cell responses after various stimuli could have given more precise answers to the possible different roles and suppressive capacity of aTregs in INR and IR.

Defining aTregs by $CD25^{high}CD147^{high}$ could be challenging (see section 4.4.3.1), yet the strong correlation with the aTregs population charachterized by the more established definition ($CD45RA^-FoxP3^{high}$), should strengthen our results. Use of this subset in the correlation analyses in **paper III** instead of $CD25^{high}CD147^{high}$ revealed similar conclusions.

5.3 Increased innate immune activation in INR

As DCs constitute an important bridge between innate and adaptive immunity, having essential antiviral functions and at the same time are implicated in chronic immune activation and induction of Tregs [196, 197, 202, 526], it is plausible that changes in DC subsets contribute to immunological disturbances seen in INR. In **paper III** we present novel data on DC phenotypes in INR. Although the fractions of DCs were comparable between INR and IR, we found increased activation of both $CD141^+$ mDCs, $CD1c^+$ mDCs and $CD303^+$ pDCs in INR compared with IR, with higher expression of HLA-DR and the co-stimulatory molecule CD86 in mDCs and expression of the maturation marker CD83 in pDCs. These activated phenotypes were not only associated to activation of $CD4^+$ and $CD8^+$ T cells and EM T cells, but also to the ratio of aTreg/rTregs, implying connection of activated DCs with both immunostimulatory and immunosuppressive changes in PLWP on ART. pDC activation and continuous IFNα secretion promotes T cell exhaustion [202] and this association would also have been relevant to explore. Higher CD86 expression in DCs are found in viremic HIV patients [211, 214], whereas the ART treated showed lower and similar levels compared to healthy controls [211]. Previous data on DC activation markers in INR are lacking.

An interesting question is which factors drive the activation of the DCs in INR? Unfortunately our study does not provide all these answers. Except for a weak correlation with sCD14, we did not detect any association with LPS or soluble inflammation markers. Still, other microbial products such as peptidoglycan, lipoteichoic acid, flagellin and unmethylated CpG rich DNA, could drive DC activation [67, 74, 205, 527]. Moreover, LPS measured in plasma correlates mostly with low-inflammatory, penta-acylated LPS [521] and might therefore not reflect translocation of pathogenic bacteria (see discussion in 5.1). As both low-level HIV and CMV viremia [528] can activate DCs and play a role in INR [94, 100], quantification of these levels would have been interesting.

There are sparse data on cellular monocyte activation in the INR patients known to have higher risk of developing non-AIDS comorbidities [298, 358, 359]. Thus, studying monocytes in this patient population is highly relevant since cellular activation of

monocytes is associated with coagulopathy and subclinical atherosclerosis even in ART-treated [160, 245, 246]. In INR we found increased HLA-DR expression in monocytes, particularly in the $CD14^{++}CD16^{+}$ (intermediate) subset, that was associated with both T cell activation and an EM T cell phenotype (**paper III)**. However, somewhat unexpectedly the proportions of the more inflammatory $CD14^{++}CD16^{+}$ (intermediate) monocytes and the expression of the other activation markers were similar in INR and IR (CD142, CD163 and CD11b). Most studies have described normalization of the fractions of intermediate monocytes in ART-treated [160, 242, 243, 529], but two short reports showed higher frequencies of intermediate monocytes in INR compared with IR [475, 530]. Differences in cohorts, such as higher number of HCV positive among the INR and a high proportion of IDU as well as dissimilar gating strategies could potentially explain the discrepancies between theirs and our findings (see section 4.4.4).

There are a considerable number of published reports on soluble markers of monocyte activation among INR. Most large studies of INR with cohort definitions comparable with our study have shown higher levels of sCD14 in INR [325, 344, 348, 475, 522], although sCD14 seems to be a less important predictor for INR status than changes in T cell phenotypes [321, 344]. Increased sCD163 was reported by Wilson *et al* [475]. In contrast, our study and other smaller studies did not detect such differences (**paper II and III)** [322, 523, 531]. Variations in proportions of IDU, co-infections with HCV and HBV could contribute to these diverging results. However, taken together most data seem to point in the direction of increased levels of sCD14 and possible also sCD163 in INR, markers that are linked to non-AIDS morbidity and mortality [109, 158, 187, 257, 258].

Few studies have investigated innate function in INR. We found that despite presence of pre-activated DCs and monocytes, the INR showed similar monocyte and DC responses after *in vitro* AT-2 HIV-stimulation as compared with IR and healthy controls. Thus, INR seem to preserve their innate immune capability to respond to HIV. A recent study showed similar HIV-specific T cell responses in INR and IR [353]. Our results do not support the hypothesis of DC dysfunction in ART-treated defined by decreased capacity to secrete IFNα and reduced induction of Th1 responses, as well as increased

production of inflammatory cytokines in response to TLR stimuli [225-228]. Our data are in line with the small study from Merlini *et al* [532], but differ from the results by Sachdeva *et al* who found reduced IFNα production in pDC enriched PBMC after TLR7 and 9 stimulation in INR compared with IR, whereas the production of pro-inflammatory cytokines were similar [225]. Differences in stimuli and laboratory settings (see section 4.3.2 and 4.4.5) may have contributed to the diverging results.

Previous data on monocyte responses in INR have been lacking. A more polyfunctional TLR induced intracellular cytokine production has been reported in monocytes from young HIV well-treated compared with healthy controls [243]. The trend of a more correlated cellular IP-10 and cytokine response in our INR cohort might point in the direction of more hyper-responsive monocytes.

Nevertheless, conclusions regarding DCs' function and monocytes' potential hyper-responsiveness in INR cannot be drawn by the present studies including ours. These innate immune cells and pDCs in particular, respond differently to various stimuli [218] and activation by several mechanisms should be studied in more detail. Furthermore, measurement of IL-12 would have given a more precise estimate of the mDC function. Some of the individuals in the INR cohort increased their CD4 count to > 400 cells/μL four years later. This heterogeneous potential for future CD4 increase in the INR cohort was also seen among those in the HIV-stimulation assays, and could have contributed to the observed lack of differences in *in vitro* responses between INR and IR.

5.3.1 IDO activity in INR

Data on IDO activity (KTR) in INR are lacking and should be explored considering its important link between innate and adaptive immunity as well as its associations with CD4 recovery [183], SNAE [83] and mortality in PLWH on ART [109, 183, 187]. In **paper II** we found no differences in the KTR between INR and IR. However, in INR patients with the most profound $CD4^+$ T cell depletion, the KTR was associated with reduced future CD4 count and CD4 gain. This is in line with the results of Byakwaga *et al* suggesting that the impact of KTR on CD4 recovery during ART was largely

determined by the extent of immune dysfunction pre-ART [183]. The KTR was also strongly correlated with aTregs in these patients, well in line with the established link between IDO activity and Tregs [66, 190]. Chen *et al* recently described a clear independent association of IDO activity with HIV DNA levels in PLWP on ART, being strongest in those with pre-ART CD4 count < 200 cells/µL [185]. This indicates a connection between IDO activity and HIV persistence that also could be relevant in INR. **Paper III** demonstrates that both healthy controls, IR and INR had similar capacity to upregulate IDO in mDCs and monocytes following *in vitro* AT-2 HIV-stimulation. However, we showed that monocytes in addition to DCs could be an important source of IDO activity in PLWH, in contrast to the findings from Boasso *et al* who did not detect upregulation of IDO in $CD14^+$ cells [189].

5.4 IP-10 - a biomarker for low CD4 recovery?

A proposed definition of a biomarker by the National Institutes of Health Biomarkers Definitions Working Group is: "A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" [533]. Already well-known predictors for incomplete immune recovery such as low nadir, pre-ART CD4 count and higher age, do not entirely explain reduced future CD4 gain (see section 1.6.3.1). This gives a rationale to search for other biomarkers linked to inadequate CD4 reconstitution, which hopefully also will highlight underlying mechanisms influencing CD4 recovery.

In **paper II** we demonstrated that the INR cohort had higher plasma IP-10 levels than the IR group and that increased IP-10 levels predicted INR status even after adjustment for age, nadir CD4 and duration of viral suppression. Furthermore, in INR with the lowest CD4 counts at inclusion, IP-10 levels were negatively associated with the CD4 count and CD4 gain two years later. To our knowledge, this is the first study reporting relation between high IP-10 levels and present INR status independent of age and nadir CD4, and a concurrent correlation with future CD4 count. As the proinflammatory IP-10 has been proposed as a biomarker in many infections, the specificity is a challenge [270,

271, 283, 284]. Importantly, exclusion of patients with previous TBC or positive Quantiferon-TBC test as indication of TBC infection, did not impact the results. Also, there were no patients with active HBV or HCV in our cohorts. Both previous research and a study published the same year as ours, report association between IP-10 levels and CD4 count both in ART treated [273, 279] and in HIV controllers [92]. In particular, persistently increased IP-10 were also related to immunological treatment failure after one year on ART [273] as well as with reduced prospective CD4 count in HIV controllers [92], increasing the generalizability of our results. However these associations were not adjusted for potential confounding factors, e.g information on HBV, HCV and TBC status contributing to increased IP-10, were lacking in the abovementioned studies.

In depth analyses in **paper III** revealed for the first time that the IP-10 increase in monocytes after the exposure to HIV *in vitro* was inversely correlated with the CD4 gain both after two and four years, with the strongest association in individuals with baseline CD4 count ≤ 300 cells/μL. Of note, an increase in IP-10 above median still predicted a lower CD4 gain after adjustment for age and nadir. However, when including duration of continuous ART, none of the parameters remained significant, but the sample size was small and therefore less robust for adjustments of several predictors (see section 4.6).

Only in INR the IP-10 response correlated with changes in cytokines in supernatants suggesting that the IP-10 increase could be accompanied by a more general immune activation in this patient group. Whether these associations are due to viral stimulation of pDCs and subsequent activation of monocytes and IP-10 expression as discussed in **paper III**, remains to be clarified. The observed association between activation of pDCs, IFNα and IP-10 levels can point in such direction [229, 278]. By attracting CXCR3 positive $CD4^+$ T cells to lymphoid tissue increased levels of IP-10 can contribute to viral transmission, increased infection of $CD4^+$ T cells and possibly also establishment of viral reservoirs [279, 534, 535]. Furthermore, IP-10 has been shown to suppress *in vitro* T cell responses in PLWH on ART, suggesting another possible disadvantageous effect of high IP-10 levels [274].

A study published early 2019 reported that the SLAMF7 receptor, which upon activation by IFNα was shown to inhibit IP-10 production from monocytes, can be “silenced” in some PLWH on ART [536]. Consequently, an activation will not reduce IP-10 secretion and these patients were also characterized by higher plasma levels of inflammatory cytokines [536]. Whether silencing of SLAMF7 contributes to increased IP-10 levels and plays a role in incomplete immune recovery, could be relevant to investigate in the future.

IP-10 is easy to quantify and levels were reported to be relatively stable intra-individually over a period of two years in a cohort of HIV controllers [92]. Yet, the levels diverge between studies and probably vary depending of the method used. Furthermore, IP-10 is increased in many inflammatory conditions making the assessment of IP-10 as a biomarker for incomplete immune recovery difficult [270, 271, 282-284]. Perez-Santiago *et al* recently found that even when categorizing INR in three groups based on various immunological T cell profiles they found no differences in prospective CD4 gain, suggesting that both other, but perhaps also more complex mechanisms cause reduced response to ART [321]. Finally, to conclude, IP-10 have to be evaluated in different and larger cohorts of INR, preferentially with a prospective design and with adjustments for comorbidities.

6. Conclusions

1. **Paper I:**

   Eight weeks of intervention with a multistrain probiotic containing *Lactobacilli* and *Bifidobacteria,* to PLWH on ART with suboptimal CD4 recovery, was well-tolerated, induced shifts in the gut microbiota consistent with the probiotic strains administered, and seemed to reduce markers of coagulation and inflammation, while no changes were detected in microbial translocation or T cell activation.

2. **Paper II and III**

   The INR cohort displayed a more activated innate and adaptive immune system compared with PLWH with normalized CD4 counts during ART. Increased activation of monocytes and DCs were associated with the disturbances seen in the T cell compartment, in particular the increased T cell activation. Despite increased spontaneous immune activation, monocytes and mDCs from INR showed strong and similar cytokine and IDO responses to *in vitro* HIV stimulation as IR and healthy controls, indicating maintained innate immune responses to HIV. In INR, the Treg population was skewed towards a higher aTregs/rTregs ratio and in INR only, the high frequencies of aTregs was associated with reduced *in vitro* HIV-specific responses. Thus, aTregs seem to be involved in impaired CD4 recovery where potential suppression of HIV-specific responses could play a role.

   Plasma IP-10 was increased in the INR cohort and together with KTR negatively associated with future CD4 recovery. More specific, *in vitro* HIV-induced IP-10 increase in monocytes at inclusion was associated with reduced CD4 gain after both two and four years. Thus, IP-10 could be a possible biomarker for incomplete restoration of the CD4 count.

7. Future perspectives

In 2016, updated WHO guidelines recommended lifelong ART to PLWH regardless of the CD4 count. This policy will not only ensure higher average CD4 levels on ART, but more important, hopefully also reduce both HIV transmission and development of AIDS and SNAE. In order to reduce the number of PLWH with incomplete CD4 recovery during ART, the currently most crucial intervention is in fact early initiation of ART. By the end of 2017, 25% of PLWH were still unaware of their HIV-status (UNAIDS.org). Upscaling of widespread testing, reaching out to high-risk groups all over the world, is an urgent strategy to improve early diagnosis of PLWH and thereby preventing advanced illness predisposing for future INR status and adverse events. Studies have also indicated that very early ART start preserves the gut immune function to a greater extent [65, 150, 537], which might contribute to less HIV-associated dysbiosis and microbial translocation.

With respect to interventions aiming to modulate the gut microbiome in PLWH on ART, it has been come increasingly clear that the gut microbiota composition varies substantially between individuals. The impact of probiotics seems to differ from person to person [519] and recent findings have also debated the probiotics beneficial effects [538]. This argues against an emipiric “one size fits all” probiotic regimen, emphasizing the probable need of a tailored, personalized approach of microbiota-directed therapies. Prebiotics, immunonutritients, FMT and dietary advices will probably be central in such an individualized approach, depending on individual microbiota signatures.

Furthermore, not only characterizing the presence of bacteria, but also enteric fungi, viruses, bacteriophage and eukaryotes, as well as the functional activity of the microbiome analyzing gene transcriptions (metatranscritomes), microbial proteins (metaproteomics) and metabolites (metabolomics) will probably help to reveal important pathways [165, 539]. One particular issue in microbiota-related studies in PLWH is the impact of sexual preference. When planning future studies, strict strategies for controlling for MSM status and other relevant confounders should be considered. Indications of a more dysbiotic gut microbiota and disturbed gut immunity in patients

with suboptimal recovery on ART, combined with promising effects seen in pilot probiotic supplement studies, give rationale for targeting this group of patients in larger, carefully designed intervention studies.

Overall, to increase the understanding of immunological pathways contributing to incomplete immune recovery, prospective studies following larger groups of PLWH at risk of INR status are needed. Comprehensive innovative technologies such as multi-omics, multiple parameter flow cytometry and supervised machine learning emerge as powerful tools for characterizing the complex mechanisms, and for the search of new biomarker signatures in INR. Changes in innate immunity are still poorly described and should be addressed, preferably including biopsies from mucosal surfaces and lymphatic tissue as the majority of innate immune cells, in particular DCs, are found here. Additionally, the role of Tregs and Tregs subsets in INR is still not clarified and functional assays of $CD4^{+}CD25^{high}CD147^{high}$ aTregs in innate and T cell responses are relevant to explore. Other studies have pointed at T cell exhaustion and death as strong predictors for INR and this should be further investigated in relation to DC activation.

To clarify whether IP-10 could serve as robust biomarker for incomplete CD4 immune recovery and risk of non-AIDS events, IP-10 should be analyzed in larger prospective HIV-cohorts from ART initiation. This should include patients with various ethnicities, co-morbidities and co-infections to validate its specificity and usefulness as biomarker. Given the strong link between IP-10 and HIV-viremia, exploring IP-10 in relation to residual viremia or HIV reservoirs in ART-treated appear to be of significant interest.

Immune-based therapies in order to improve prognosis in ART-treated and in particular INR, are becoming increasingly relevant. In addition to PD-1 blockade which now is being investigated in INR, targeting activated and highly suppressive Tregs rather than all Tregs, or IP-10 blockade could be potentially interesting strategies.

In light of the increasing evidence for low CD4/CD8 ratio as an important predictor for non-AIDS related events [365, 366] and mortality [364], future studies should also focus on PLWH with persistent reduced CD4/CD8 ratios despite adequate CD4 recovery during ART.

Presently, ensuring adherence to ART, modifying lifestyle factors related to development of SNAE, such as smoking cessation, physical activity and dietary advice, and diagnosing and optimizing treatment of comorbidities as for instance hypertension and diabetes, are currently our best tools to improve the overall prognosis for patients with incomplete immune recovery.

8. References

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III

Activated dendritic cells and monocytes in HIV immunological nonresponders: HIV-induced interferon-inducible protein-10 correlates with low future CD4^{+} recovery

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Objective: To explore monocyte and dendritic cell immune responses, and their association with future CD4^{+} gain in treated HIV patients with suboptimal CD4^{+} recovery.

Design: A cross-sectional study of HIV-infected, virally suppressed individuals on antiretroviral therapy for at least 24 months; 41 immunological nonresponders (INRs) (CD4^{+} cell count <400 cells/μl) and 26 immunological responders (CD4^{+} cell count >600 cells/μl). Ten HIV-infected antiretroviral therapy-naive and 10 HIV-negative healthy persons served as controls. CD4^{+} cell counts were registered after median 2.4 and 4.7 years.

Methods: Monocyte, dendritic-cell and T-cell activation and regulatory T cells (Tregs) were analyzed by flow cytometry. In INR and immunological responder subgroups matched on age and nadir CD4^{+} cell count, upregulation of interferon-inducible protein-10 (IP-10) and indoleamine 2,3-dioxygenase in monocytes and dendritic cells and cytokines in cell supernatants were measured *in vitro* in peripheral blood mononuclear cells stimulated with aldrithiol-2-inactivated HIV-1.

Results: The INR group displayed higher spontaneous activation of both monocytes (HLA-DR^{+}) and myeloid and plasmacytoid dendritic cells (HLA-DR^{+}, CD83^{+} and CD86^{+}) compared with immunological responders, and this was associated with increased T-cell activation (CD38^{+}HLA-DR^{+}), an effector memory T-cell phenotype and activated Tregs. The IP-10 response in monocytes after in-vitro HIV stimulation was negatively associated with prospective CD4^{+} gain. IP-10, indoleamine 2,3-dioxygenase and cytokines levels were comparable between the groups, but inversely correlated with activated Tregs in INRs.

Conclusion: HIV-infected individuals with suboptimal immune recovery demonstrated more activated monocytes and in particular dendritic cells, compared with patients with acceptable CD4^{+} gain. A high level of HIV-specific IP-10 expression in monocytes may be predictive of future CD4^{+} recovery.

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Introduction

Despite modern antiretroviral therapy (ART) and persistent viral suppression, 15–30% of people living with HIV (PLWH) do not normalize their $CD4^+$ cell count, denoted immunological nonresponders (INRs) [1–4]. Low nadir $CD4^+$ cell count, long duration of HIV-infection before ART, coinfections such as hepatitis C and older age are well known factors associated with an incomplete immune recovery [2,4–6]. INRs have increased chronic immune activation and inflammation, which probably contribute to the higher morbidity and mortality seen in this group [7–11]. Given the substantial proportion of PLWH who still initiates ART in late disease, INRs will continue to be of clinical relevance [4,12].

The underlying immunological causes of incomplete immune recovery are multifactorial. Several studies have shown a more activated and differentiated T-cell phenotype [9,13–16] as well as higher percentages of activated regulatory T cells (aTregs) [10,15,16] in INRs. We have recently published increased levels of plasma interferon-inducible protein-10 (IP-10) in an INR cohort, in which IP-10 and kyurenine/tryptophan ratio, a measure of indoleamine 2,3-dioxygenase (IDO) activity, were negatively associated with the $CD4^+$ cell count after 2 years [16]. IP-10 is mainly produced by monocytes and myeloid dendritic cells (mDCs) in HIV-negative persons after in-vitro HIV-1 stimulation [17] and is also associated with monocyte activation in HIV-infection [18]. Increased activation of monocytes is reported even in ART-treated PLWH and is related to cardiovascular disease and markers of inflammation and coagulation [19–23]. IDO-activity predicts mortality in treated HIV-infection [24] and is expressed in mDCs and plasmacytoid dendritic cells (pDCs) both in simian immunodeficiency virus (SIV)-infected macaques [25] and in HIV-negative humans after *in vitro* stimulation with inactivated HIV [26,27], or lipopolysaccharide (LPS) in combination with interferon-gamma (IFN-γ) [28].

Dendritic cells serve as a bridge between innate and adaptive immunity, being major drivers of Th1 responses and persistent IFNα secretion which have antiviral functions, but also contribute to chronic immune activation. Furthermore, dendritic cells upregulate IDO and induce Tregs that could both reduce harmful, general inflammation and dampen beneficial HIV-specific immune responses [29–31]. Even virally suppressed PLWH have signs of dendritic cell dysregulation as some demonstrate subnormal dendritic cell counts in blood [32–36], weakened pDC IFNα secretion after exogenous stimuli [36,37] and impaired mDC induction of Th1 responses [38].

To our knowledge, few studies have investigated monocytes and dendritic cells in INRs. Increased proportion of intermediate monocytes [39,40] and lower absolute pDC count with reduced IFNα production have been reported in INRs compared with PLWH with normalized $CD4^+$ cell count [41].

We set out to study activation of monocytes and dendritic cells in INRs compared with immunological responders, ART-naive PLWH and healthy controls, and in-vitro HIV-specific monocyte and dendritic cell responses in INR and immunological responder subgroups matched on age and nadir $CD4^+$ cell count. We hypothesized that INRs had more activated monocytes and dendritic cell subsets and higher in-vitro production of IP-10, IDO and cytokines than the immunological responder group that might contribute to an inadequate future immune reconstitution in INRs.

Methods

Study participants

Forty-one virally suppressed HIV-infected INRs with $CD4^+$ cell count less than 400 cells/μl and 26 immunological responders with $CD4^+$ cell count more than 600 cells/μl were recruited between October 2012 and April 2013 as previously reported [16]. Both groups had received continuous ART for at least 24 months with HIV-RNA 20 copies/ml or less for the last 18 months. $CD4^+$ cell counts were obtained from fresh samples and recorded at baseline and median 2.4 and 4.7 years after inclusion. The last routine $CD4^+$ cell counts that were available prior to data analyses of the previous [16] and the present reports were used. For comparison, 10 ART-naive individuals with duration of HIV-infection at least one year and 10 HIV-negative healthy controls, all age and sex matched, were included. Peripheral blood mononuclear cells (PBMCs) and EDTA plasma were sampled from all participants at inclusion, frozen and stored for later analyses. All participants provided written informed consent. The study was approved by the Regional Ethics Committee (1.2007.83 and 2015/629).

Flow cytometry analyses of ex-vivo monocyte and dendritic cell activation

Flow cytometry analyses were performed on thawed PBMCs. After 2 h rest, one million (viability >85%) PBMCs were incubated with Fc block (BD Biosciences, San Jose, California, USA) prior to staining with surface markers for 15 min in room temperature. The cells were fixated in 1% BD CellFIX (BD Biosciences) before acquisition on BD FACSCanto II (BD Biosciences). The fluorochrome-conjugated antibodies for the monocyte and dendritic cell panels are listed in Table S1, http://links.lww.com/QAD/B453. Results were analyzed with the FlowJo software version 10.4.1 (Tree Star Inc, Ashland, Oregon, USA). As staining controls, all antibodies in the other channels were combined with concentration matched isotypes for the activation markers, and fluorescence minus one (FMO) was used for anti-CD83. The gating strategy is shown in Fig. S1,

http://links.lww.com/QAD/B453. Monocyte subsets were defined as $CD45^+HLA\text{-}DR^+Lineage^-Fixable\ Viability^-$ and either $CD14^{++}CD16^-$ (classical), $CD14^{++}CD16^+$ (intermediate) or $CD14^+CD16^{++}$ (nonclassical). Dendritic cells were characterized as $CD45^+HLA\text{-}DR^+Lineage^-FixableViability^-$ and further subdivided into $CD1c^+$ mDCs, $CD141^{++}$ mDCs or $CD303^+$ pDCs.

Intracellular interferon-inducible protein-10 and indoleamine 2,3-dioxygenase detection after in-vitro stimulation with inactivated HIV-1

Owing to the strong association of nadir $CD4^+$ with low $CD4^+$ recovery, 20 INRs and 20 immunological responders with comparable age and nadir $CD4^+$ cell count, and eight age and sex-matched healthy controls were selected for in-vitro HIV-stimulation analyses (Table S2, http://links.lww.com/QAD/B453). One million thawed PBMCs (viability >90%), were cultured for 18 h at 37 °C, 5% CO_2 in 200 μl RPMI 1640 (Lonza, Verviers, Belgium) containing 10% heat-inactivated fetal calf serum and penicillin/streptomycine/L-glutamine, with either aldrithiol-2-inactivated (AT-2) HIV-1 (dual-tropic; X4 and R5, lot P4311) at a final concentration of 500 ng/ml p24 [42], or microvesicles with equivalent protein concentration (mock) as negative control [43]. Brefeldin A 1 μl/ml (BD Biosciences) was added after 6 h. Following stimulation supernatants were harvested and the cells stained for either monocyte or dendritic cell surface markers as previously described (Table S1, http://links.lww.com/QAD/B453), subsequently fixated and permeabilized using BD Cytofix/Cytoperm (BD Biosciences) and stained intracellularly for IP-10 and IDO (Table S1, http://links.lww.com/QAD/B453). All IP-10 and IDO responses were calculated with subtraction of the corresponding mock stimulated control. For gating strategy see Fig. S2, http://links.lww.com/QAD/B453.

ELISA and multiplex analyses

Soluble (s)CD163 was analyzed in INRs and immunological responders from snap frozen EDTA plasma in duplicate by immunoassay (DY1607; R&D Systems, Minneapolis, Minnesota, USA) according to the manufacturer's instructions. The intraassay and interassay coefficients of variation (CV) were less than 10%.

Concentrations of IL-1β, IL-1 receptor antagonist (IL-1ra), IL-6, IL-10, IL-18, IFNα2, IFNγ, tumor necrosis factor, IP-10 and macrophage inflammatory protein 1beta (MIP-1β)/CCL4 were measured in thawed supernatants (10 000 g/10 min/4 °C) by nine-plex and single-plex (Bio-Rad, Oslo, Norway) assays, respectively. The samples were diluted 1 : 7.5 and 1 : 75 as appropriate, analyzed with a Luminex IS 100 instrument (Bio-Rad, Hercules, California, USA) according to instructions from the manufacturer and run in duplicates. Both intraassay and interassay CV were less than 10%. All AT-2 HIV virus responses were found by subtracting concentrations from the corresponding mock stimulated sample.

Multiplex and ELISA analyses of other soluble markers in plasma and flow cytometry phenotyping of T-cell subsets including Tregs have been previously presented [16], and are only used in correlation analyses in the current article. The methods are described in brief in the Supplementary methods, http://links.lww.com/QAD/B453.

Statistical analyses

Statistical analyses and graphical presentations were performed by SPSS statistics 25 (IBM Corp., Armonk, New York, USA) and GraphPad Prism V7.04 software (GraphPad, San Diego, California, USA) using nonparametric statistics. To reduce the percentage of CV (%CV) in the flow cytometry experiments caused by small cell populations, populations consisting of less than 100 cells were excluded for further analyses of percentages of activation markers and when less than 50 cells also for determination of median fluorescence intensity (MFI) [44]. For comparison between more than two groups Kruskal–Wallis test followed by Dunn's post-hoc test with correction for multiple comparisons were applied. Mann–Whitney *U* test was used when analyzing two groups, Wilcoxon test for paired samples, Fisher's exact test or Pearson chi-squared test for categorical variables and Spearman's rank correlation for correlation between parameters. Factors associated with prospective $CD4^+$ gain were further analyzed with binary logistic regression adjusting for age, nadir $CD4^+$ cell count and duration of ART. The outcome variable was dichotomized on median within the actual patient group. A two-tailed significance level of 0.05 was set.

Results

Characteristics of the study participants

The baseline characteristics for the INR and immunological responder cohorts have previously been described in detail [16]. The ART-naive had median $CD4^+$ cell count 553 (interquartile range 346–667) cells/μl, $CD4^+/CD8^+$ ratio comparable with INRs, shorter duration of HIV infection ($P = 0.004$) and higher nadir $CD4^+$ cell count ($P < 0.001$) than both INRs and immunological responders (Table 1). In the subgroups matched on age and nadir $CD4^+$, selected for AT-2 HIV stimulation assay, the INR group ($n = 20$) had lower viral load at initiation of continuous ART ($P = 0.02$) and shorter duration of both viral suppression ($P = 0.01$) and ART use ($P = 0.01$) compared with the immunological responder group ($n = 20$) (Table S2, http://links.lww.com/QAD/B453).

Table 1. Characteristics of the study cohort at inclusion.

Total study population	INR, $n=41$	IR, $n=26$	ART-, $n=10$	HC, $n=10$	P value*
Age (IQR)	49.9 (41.6–57.9)	45 (39.3–53.8)	50.6 (39.6–59.4)	47.2 (43.5–58.0)	NS
Male sex, *n* (%)	35 (85.4)	18 (69.2)	7 (70)	7 (70)	NS
Ethnicity, *n* (%)					
White	29 (70.7)	17 (65.4)	5 (50)	10 (100)	NS
Risk group, *n* (%)					
MSM	22 (53.7)	13 (50)	1 (10)**		**0.04**
Other[a]	19 (46.3)	13 (50)	9 (90)**		**0.04**
Comorbid diseases, *n* (%)					
Cardiovascular	6 (14.6)	0 (0)	2 (20)		NS
Any comorbidity[b]	21 (51.2)	6 (23.1)	5 (50)		NS
CMV IgG pos	41 (100)	25 (96.2)	9 (90)		NS
HIV characteristics (IQR)					
Years since HIV diagnosis	8.6 (6.4–15.2)	9.2 (7.0–14.1)	3.6 (2.2–6.8)**,***		**0.004**
Years of continuous ART	5.5 (3.1–6.7)	6.6 (4.4–8.7)			NS
Viral load at ART initiation (copies/ml)	67 500 (29 000–110 000)	100 000 (50 000–330 000)			NS
Duration of viral suppression (years)	3.8 (2.0–5.8)	6.1 (4.0–7.6)			**0.01**
Viral load at inclusion (copies/ml)	≤20	≤20	36 000 (19 750–128 000)**,***		**<0.001**
$CD4^+$ cell count nadir (cells/μl)	100 (20–157)	180 (120–220)	369 (346–512)**,***,****		**<0.001**
$CD4^+$ cell count at inclusion (cells/μl)	285 (232–348)	810 (740–864)	553 (346–677)**,****		**<0.001**
$CD8^+$ cell count at inclusion (cells/μl)	670 (521–890)	1005 (820–1587)	1257 (973–2491)**,****		**<0.001**
$CD4^+/CD8^+$ at inclusion	0.43 (0.32–0.57)	0.79 (0.63–0.99)	0.35 (0.25–0.74)***,****		**<0.001**

Data are presented as no. (%) of study participants or median IQR values. ART, antiretroviral therapy; ART-, antiretroviral therapy naive HIV-infected; CMV, cytomegalovirus; HC, healthy control; INR, immunological nonresponder; IQR, interquartile range; IR, immunological responder.
[a]Other. Heterosexual or unknown. There were no intravenous drug abusers.
[b]One or more of the following comorbidities; cardiovascular disease, hypertension, diabetes, renal disease, osteoporosis, chronic obstructive pulmonary disease, neurodegenerative disease, previous cancer or *Mycobacterium tuberculosis* infection.
**P* values for Kruskal–Wallis test or Pearson Chi-squared test for comparison between multiple groups, Mann–Whitney *U* test for comparison INR vs. IR. Significant values are shown in bold.
***P* less than 0.05 for comparison INR vs. ART-. Fisher's exact test or Dunn's post-hoc test.
****P* less than 0.05 for comparison IR vs. ART-. Dunn's post-hoc test.
*****P* less than 0.05 for comparison INR vs. IR. Dunn's post-hoc test.

A median of 4.7 years after inclusion the median $CD4^+$ gain was 55 (−9 to 115) cells/μl among INRs and 37% had reached a $CD4^+$ cell count above 400 cells/μl. However, only three persons achieved $CD4^+$ cell count above 500 cells/μl. The median increase in $CD4^+$ from 2.4 until 4.7 years after inclusion was 18 (−29 to 75) cells/μl and 58% of the INRs increased their $CD4^+$ cell count in this period. Nevertheless, the $CD4^+$ cell counts stabilized the last 12 months of follow-up (Fig. S3, http://links.lww.com/QAD/B453). Duration of continuous ART and viral suppression, nadir $CD4^+$ and age were all related to prospective $CD4^+$ cell count and/or $CD4^+/CD8^+$ ratio (Table S3, http://links.lww.com/QAD/B453).

Activated monocytes in immunological nonresponders correlate with T-cell activation and low $CD4^+$ cell counts

We first analyzed the phenotypes of the various monocyte subsets. In the INR group, the frequency of monocytes was negatively associated with the $CD4^+$ cell count at inclusion ($r=-0.39$, $P=0.0012$), and the monocytes constituted a higher fraction of the $CD45^+$ cells within the INR than in the immunological responder group (Fig. S3, http://links.lww.com/QAD/B453). The INRs demonstrated increased MFI of HLA-DR in monocytes compared with immunological responders ($P=0.043$), particularly in the $CD14^{++}CD16^+$ subset ($P=0.013$) (Fig. 1a). The HLA-DR expression correlated with T-cell activation, an effector memory (EM) T-cell phenotype and negatively with baseline $CD4^+$ cell count and $CD4^+/CD8^+$ ratio ($r=-0.35$, $P=0.004$) (Table S4, http://links.lww.com/QAD/B453). Overall, the ART-naive had the highest CD16 expression in monocytes ($P=0.002$) (Fig. S4, http://links.lww.com/QAD/B453). However, we found no differences between the HIV groups neither in the distribution of the various monocyte subsets, nor in the MFI values or frequencies of cells expressing the activation markers CD142 [tissue factor (TF)], CD163 or CD11b on these subsets (Fig. S4, http://links.lww.com/QAD/B453 and Fig. S5, http://links.lww.com/QAD/B453). Nevertheless, all HIV-positive groups showed higher MFI of CD142 and lower MFI and fractions of $CD163^+$ monocytes than healthy controls (Fig. S5, http://links.lww.com/QAD/B453). The expression of CD163 or percentages of $CD163^+$

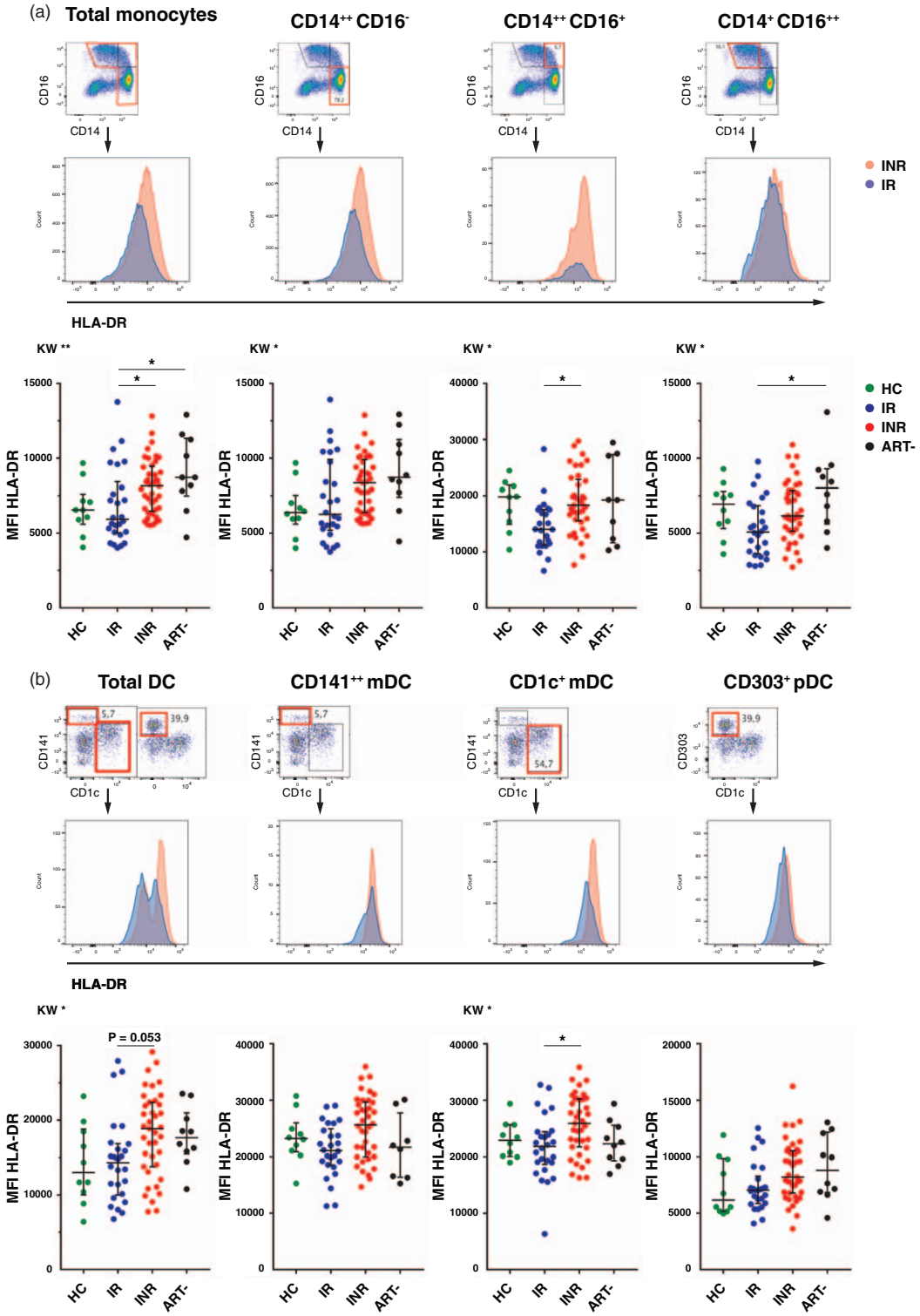

Fig. 1. HLA-DR expression in monocyte and dendritic cell subsets in the different cohorts. Parts (a) and (b) show overlay plots of median fluorescence intensity of HLA-DR in one immunological nonresponder and one immunological responder for various monocyte and dendritic cell subsets, respectively. The graphs display the differences among healthy control, immunological responder, immunological nonresponder and antiretroviral therapy-naive groups in the HLA-DR expression in the specific monocyte (a) and dendritic cell (b) subsets. Kruskal–Wallis test followed by Dunn's post-hoc test for correction for multiple comparisons. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Lines indicate median and interquartile range. ART-, antiretroviral therapy naive HIV-infected; DC, dendritic cells; HC, healthy control; INR, immunological nonresponder; IR, immunological responder; MFI, median fluorescence intensity.

monocytes were not associated with any inflammation markers, T-cell activation or Tregs. Furthermore, analysis of plasma sCD163 revealed no difference between INRs and immunological responders and no correlation with cellular CD163 expressed as MFI or frequencies of $CD163^+$ monocytes.

Dendritic cell activation is increased in immunological nonresponders and correlates with T-cell activation and low $CD4^+$ cell counts

We then investigated the distribution of pDCs and mDCs in the various cohorts. The ART-naive had lower fractions of total dendritic cells compared with both INRs and healthy controls ($P = 0.008$ and $P < 0.001$), but there were no differences between INRs and immunological responders (Fig. S4, http://links.lww.com/QAD/B453). Overall, all HIV groups had more activated dendritic cells than the controls. Still, there was a higher level of dendritic cell activation in the INR compared with the immunological responder group (Figs. 1b and 2b–d) with higher MFI of HLA-DR in the total dendritic cell population, mainly driven by increased expression in $CD1^+$ mDCs ($P = 0.021$) (Fig. 1b). Furthermore, the expression of the maturation marker CD83 in pDCs, the fraction of $CD83^+$ pDCs and $CD83^+CD141^+$ mDCs (Fig. 2b), the expression of the T-cell costimulatory molecule CD86 in both mDC subsets (Fig. 2c) and the fraction of $CD83^+CD86^+CD141^+$ mDCs (Fig. 2d) were all higher in INRs than immunological responders. Finally, activation of pDCs and mDCs correlated strongly with activated T cells, to a lesser degree with the proportion of aTregs/resting (r)Tregs, and all the activated dendritic cell subsets were negatively associated with the baseline $CD4^+$ cell counts (Table S4, http://links.lww.com/QAD/B453). There were no relation between dendritic cell activation and soluble inflammation markers except for a weak correlation between soluble (s)CD14 and HLA-DR expression in $CD1c^+$mDC and CD83 in pDCs ($r = 0.30$, $P = 0.016$, and $r = 0.32$, $P = 0.011$).

HIV-induced upregulation of interferon-inducible protein-10 and indoleamine 2,3-dioxygenase in monocytes and myeloid dendritic cells *in vitro* are comparable in immunological nonresponders, immunological responders and controls

Next, we analyzed the effect of exposure to AT-2 HIV *in vitro* upon the various mDC and monocyte subsets in the INRs, immunological responders and healthy controls. There was a significant upregulation of both MFI of IDO and IP-10 and frequencies of IP-10 positive monocyte and mDC subsets compared with mock-stimulated samples in all cohorts (Fig. 3), with the exception of MFI of IDO in $CD141^+$ mDCs, which decreased. The fraction of IP-10 positive cells increased most in monocytes, whereas IDO expression was highest in mDCs. We found no significant differences between the INR, immunological responder and healthy control groups neither in frequencies of $IP\text{-}10^+$ cells, nor in the MFI values of IP-10 or IDO in monocytes and mDCs after AT-2 HIV stimulation (Fig. 3).

High fractions of activated regulatory T cells are associated with reduced in-vitro HIV-specific responses in immunological nonresponders

We observed clear cytokine responses after AT-2 HIV stimulation, although we did not detect any significant differences between the INRs, immunological responders and healthy controls (Fig. S6, http://links.lww.com/QAD/B453). Changes in soluble IP-10 levels correlated strongly with IP-10 measured by flow cytometry in both monocytes (ΔMFI $r = 0.55$, $P < 0.001$, $\Delta\%$ $r = 0.63$, $P = 0.001$) and mDCs (ΔMFI $r = 0.47$, $P = 0.002$, $\Delta\%$ $r = 0.32$, $P = 0.044$), suggesting that the IP-10 in the supernatants could originate from both monocytes and mDCs. In the INR subgroup the IP-10 expression in monocytes and/or dendritic cells analyzed by flow cytometry corresponded to changes in soluble IFNα2, IL-1ra, IL-18, IFNγ and MIP-1β (Table S5, http://links.lww.com/QAD/B453).

Furthermore, in INRs, the upregulation of both IP-10 and IDO in monocytes and mDCs, as well as the IFNα2, IL-18 and IFNγ responses after exposure to AT-2 HIV, were strongly inversely correlated with the proportion of aTregs (Fig. 4a–c). In immunological responders, there were no such associations.

HIV-specific monocyte interferon-inducible protein-10 responses are negatively associated with future $CD4^+$ gain

Finally, we aimed to explore markers associated with future $CD4^+$ gain in the INR group. The increase of IP-10 expression in monocytes observed after exposure to AT-2 HIV *in vitro*, was negatively correlated with the $CD4^+$ gain seen 2.4 and 4.7 years later (Fig. 4d). In INR with $CD4^+$ cell count 300 cells/μl or less at baseline this association was even stronger ($r = -0.86$, $P = 0.007$) and there was also a negative correlation between the MFI IP-10 response and the prospective $CD4^+/CD8^+$ ratio ($r = -0.71$, $P = 0.047$). When adjusting for age and nadir $CD4^+$, an increase in MFI of IP-10 in monocytes above median, could still predict a lower $CD4^+$ gain in INRs after both 2.4 and 4.7 years [2.4 years; odds ratio (OR) = 19.2 (95% confidence interval (CI): 1.1–350.2), $P = 0.046$ and 4.7 years; OR = 21.2 (95% CI: 1.1–399.6), $P = 0.041$]. Nevertheless, when including duration of continuous ART in the model, none of the parameters remained significant.

Discussion

We have explored innate immunity in an INR cohort compared with ART-treated PLWH with satisfactory $CD4^+$ recovery, by characterizing monocyte and

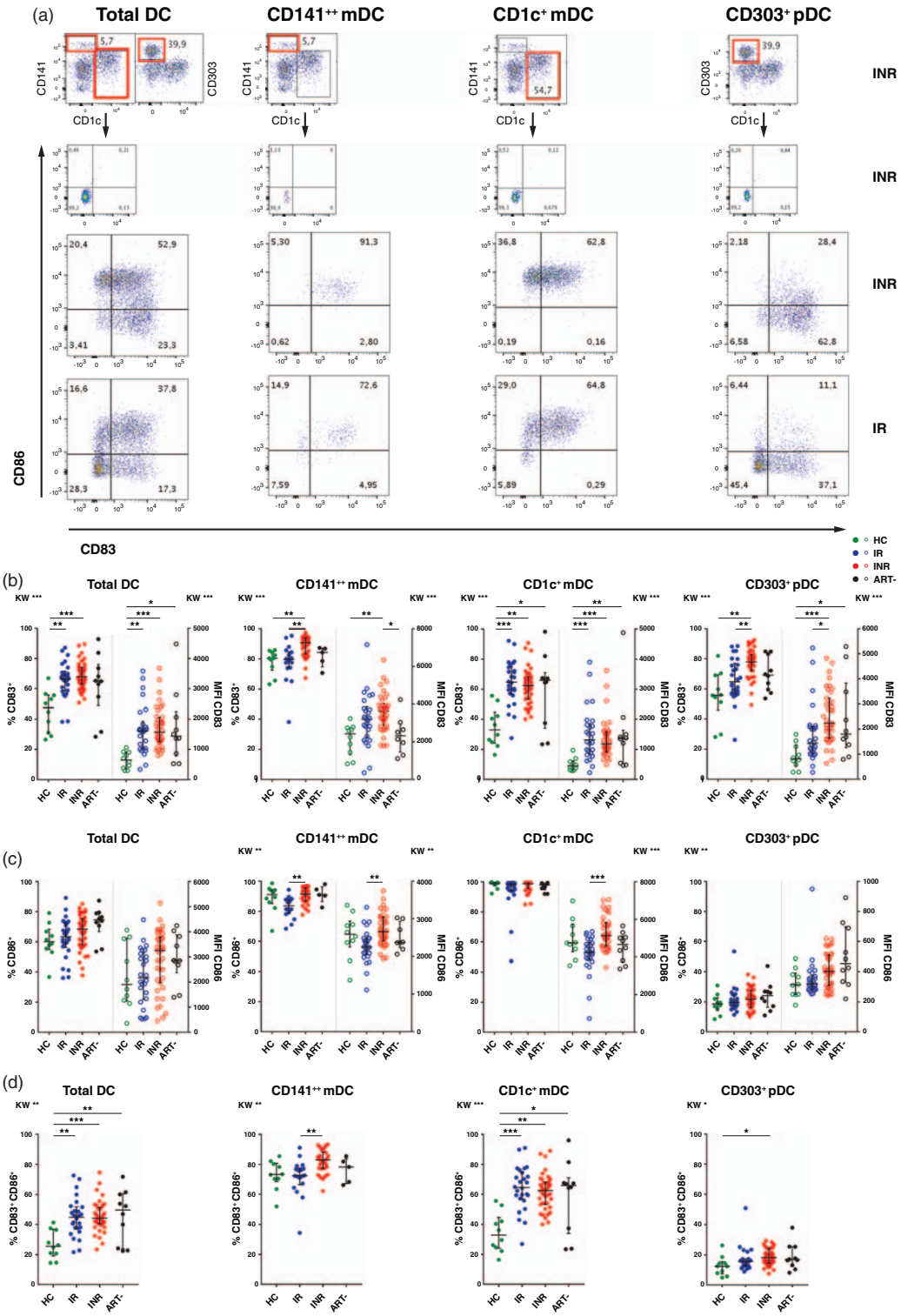

Fig. 2. Dendritic cell activation markers in the various groups. Part (a) illustrates gating strategy of dendritic cell activation markers based on isotype or fluorescence minus one in total dendritic cells and the different dendritic cell subsets. The plots show representative examples from one immunological nonresponder and one immunological responder. In parts (b), (c) and (d), the graphs display both percentage (solid dots) and median fluorescence intensity (open dots) values of the activation markers CD83, CD86 and the coexpression of CD83 and CD86 in the various dendritic cell subsets for each group. Kruskal–Wallis test followed by Dunn's post-hoc test for correction for multiple comparisons. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Lines indicate median and interquartile range. ART-, antiretroviral therapy naive HIV-infected; DC, dendritic cells; HC, healthy control; INR, immunological non-responder; IR, immunological responder; MFI, median fluorescence intensity.

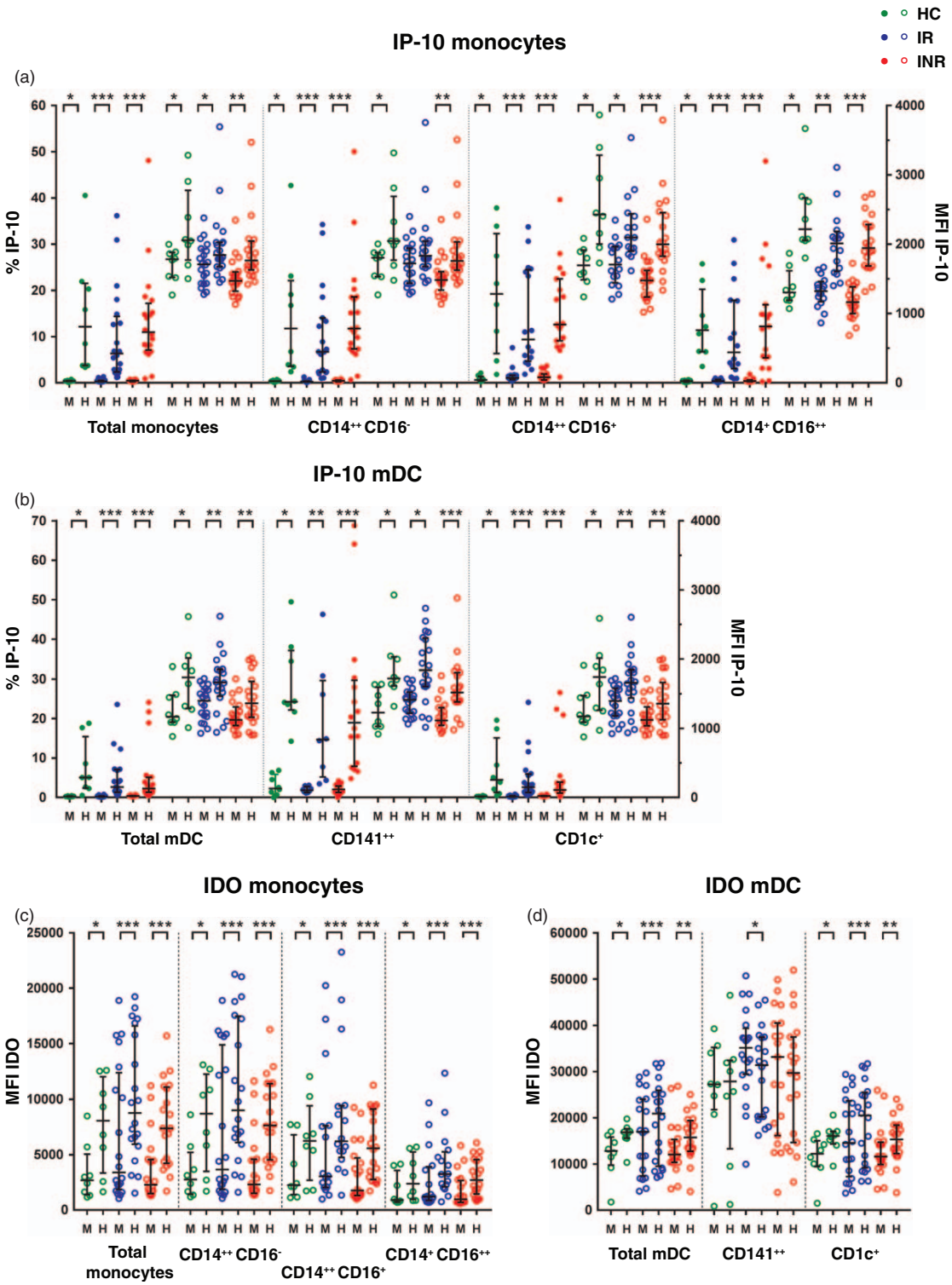

Fig. 3. Interferon-inducible protein-10 and indoleamine 2,3-dioxygenase responses in monocytes and myeloid dendritic cells after exposure to aldrithiol-2 inactivated HIV-1 *in vitro*. Percentages (solid dots) and median fluorescence intensity (open dots) of interferon-inducible protein-10 in monocytes (a) and myeloid dendritic cells (b) after in-vitro stimulation of peripheral blood mononuclear cells with either mock (M) or aldrithiol-2 inactivated HIV-1 (H) in healthy controls, immunological responders and immunological nonresponders. Parts (c) and (d) display indoleamine 2,3-dioxygenase responses in monocytes and myeloid dendritic cells, respectively. Wilcoxon test for pairwise comparisons between mock and aldrithiol-2 inactivated HIV-1 stimulation. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Lines indicate median and interquartile range. HC, healthy control; INR, immunological nonresponder; IR, immunological responder; mDC, myeloid dendritic cell; MFI, median fluorescence intensity.

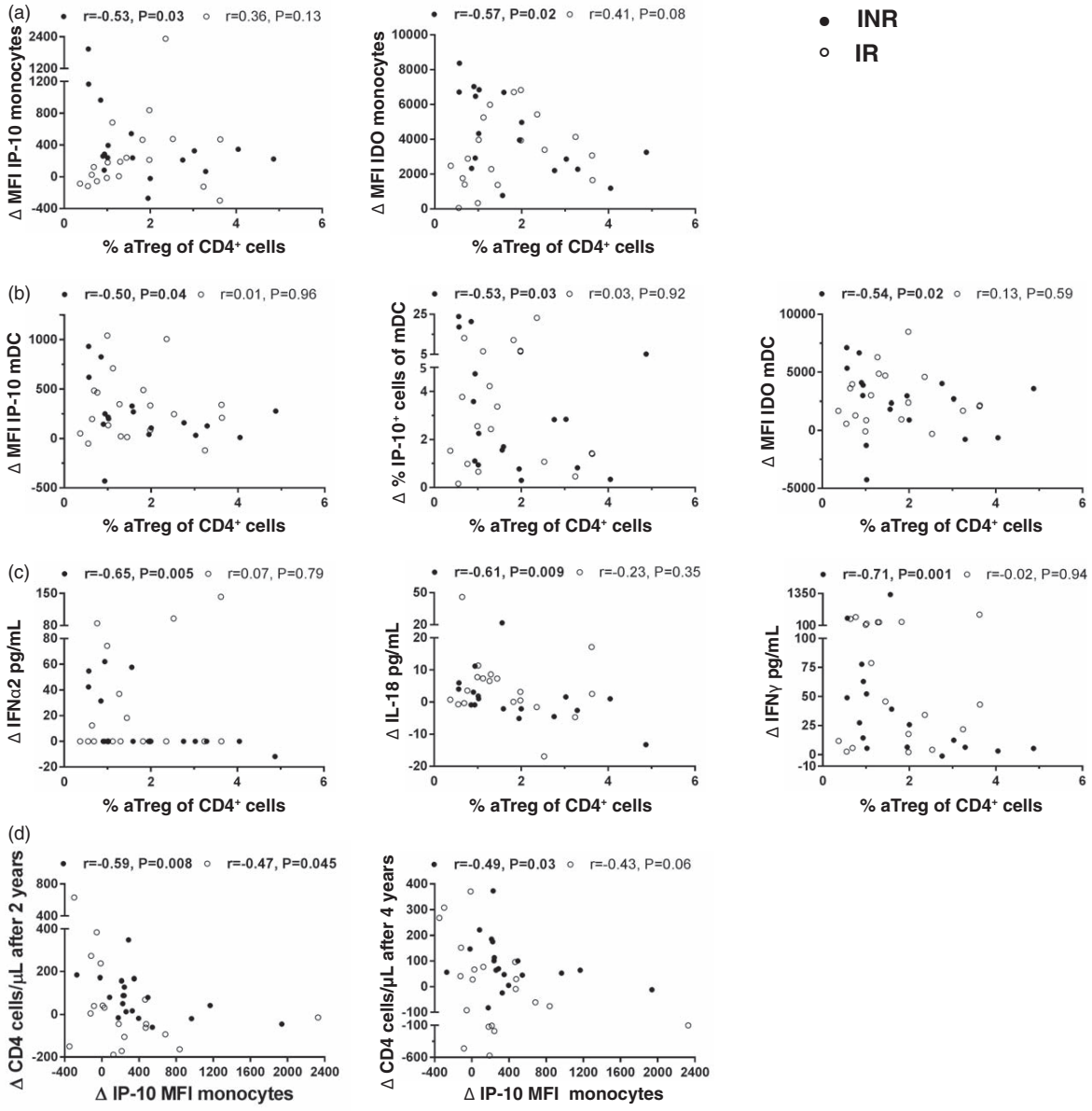

Fig. 4. Associations between fraction of activated regulatory T cells and interferon-inducible protein-10, indoleamine 2,3-dioxygenase and cytokine upregulation after exposure to aldrithiol-2 inactivated HIV-1 *in vitro* and correlation between interferon-inducible protein-10 responses in monocytes and CD4$^+$ recovery after 2 and 4 years. Correlation between percentages of activated regulatory T cells and indoleamine 2,3-dioxygenase and interferon-inducible protein-10 responses in monocytes (a) and myeloid dendritic cells (b) and cytokine responses in supernatants (c) after in-vitro stimulation with aldrithiol-2 inactivated HIV-1. (d) Associations with interferon-inducible protein-10 increase in monocytes after exposure to HIV *in vitro* and prospective CD4$^+$ gain after 2 and 4 years. Spearman rank order correlation. The aldrithiol-2 inactivated HIV-1 responses are calculated with subtraction of the mock stimulated control. •INR, immunological nonresponder; ○IR, immunological responder; aTregs, activated regulatory T cells (%CD147^{++}CD25^{++} of CD4$^+$); mDC, myeloid dendritic cell; MFI, median fluorescence intensity.

dendritic cell subsets and responsiveness to in-vitro HIV stimulation and their associations with T-cell phenotypes. To our knowledge, this is the first study that presents such thorough characterization of phenotypes and functionality in innate and adaptive immunity in a group of INRs. Our main findings can be summarized as follows: first, INRs demonstrated increased activation of dendritic cells and monocytes compared with immunological responders. Second, these changes were associated with T-cell activation, a shift towards an EM T-cell phenotype and higher frequency of aTregs. Third, in INRs, the presence of aTregs was correlated with reduced in-vitro

HIV-specific IDO and cytokine responses, and fourth, a high HIV-specific increase in IP-10 expression in monocytes was associated with lower $CD4^+$ recovery after 2 and 4 years.

mDCs are potent antigen-presenting cells and recognize diverse pathogens due to their broad expression of Toll-like receptors (TLRs) [30]. Following TLRs engagement, mDCs upregulate major histocompatibility complex class II, the costimulatory molecules CD80 and CD86, and produce IL-12 which induces Th1 cell responses [30,45]. In our cohorts, INRs showed increased CD86 and HLA-DR expression in mDCs compared with the immunological responder group. Activated pDCs are the most potent producers of IFNα [46] which stimulate a wide range of immune cells. The maturation marker CD83 was higher expressed in pDCs in the INR cohort than in immunological responders and HIV-negative controls. Furthermore, we found that the activated dendritic cells were associated with T-cell activation, an EM T-cell phenotype, as well as the fraction of aTregs/rTregs, and inversely with the $CD4^+$ cell count. Our results thereby suggest that activation of dendritic cells is connected to well known disturbances in the T-cell compartment in both immune-stimulatory and immune-suppressive directions.

HIV and other viruses can activate pDCs directly [34,45,47], whereas mDCs seem to be dependent of exposure to pDC-derived cytokines to mature after exposure to HIV [34,45]. In HIV-infected individuals also LPS, other pathogen-derived factors and unmethylated DNA contribute to activation of mDCs and pDCs, respectively [30,48,49]. We found no correlations between dendritic cell activation and soluble inflammation markers in plasma except for a weak correlation with sCD14. Despite lack of association with LPS, dendritic cell activation could still be driven by other microbial products [47,49,50]. Of note, none of the patients had acute infections, hepatitis C or B viremia, but with one exception, all were cytomegalovirus (CMV)-positive and differences in CMV replication and low-level HIV viremia might be partly responsible for the activation of dendritic cells [51].

Contrary to a recent report [39], we discovered higher HLA-DR expression in monocytes in INRs compared with immunological responders. Bandera *et al.* studied frequencies of HLA-DR^+ $CD14^+$ cells and as most monocytes express HLA-DR, such differences could be more difficult to detect. Variations in definition and gating strategies of monocytes and different patient populations can also be a relevant explanation of our diverging results. As seen for dendritic cells, activated monocytes correlated inversely with the $CD4^+$ cell count and were associated with T-cell activation, but not with soluble inflammation markers linked to non-AIDS morbidity in ART-treated PLWH. Nevertheless, in line with two recently published studies, all our HIV groups showed higher expression of TF than controls, which are related to coagulation and cardiovascular disease [22,23].

Despite the increased spontaneous ex-vivo activation of monocytes and dendritic cells seen in INRs, there were no differences between INRs, immunological responders and controls in the in-vitro cell upregulation of IP-10 and IDO or cytokine levels in supernatants. This implies that innate immunity in INRs preserve their capacity to respond to the HIV virus. Our data support other studies reporting similar cytokine production in supernatants in INRs and immunological responders after exposure to single-stranded RNA [52], TLR7 or TLR9 [41], yet IFNα synthesis seemed to be reduced in INRs [41]. Few had a measurable raise in IFNα2 in our study and the numbers are thus too low to draw any conclusions. In line with Simmons *et al.* [17], we found that monocytes had the highest fractions of IP-10 positive cells after AT-2 HIV stimulation. However, in contrast to Boasso *et al.* [26], we saw an increase in IDO expression in both monocytes and mDCs indicating that also monocytes could be an important source for IDO activity in HIV infection.

Although the increases in cytokines in supernatants and cellular IP-10 and IDO were similar, the inflammatory responses seemed to be more closely correlated in INRs compared with immunological responders. Only in INRs, the upregulation of IP-10 in monocytes and mDCs were related to the increase in several supernatant cytokines. This could imply that IP-10 responses are accompanied by a more general and possible harmful immune activation in patients with incomplete immune recovery. Moreover, we identified a negative association between the frequency of aTregs and the upregulation of several cytokines and cellular IDO and IP-10 in INRs, suggesting that presence of aTregs may suppress HIV-specific responses in patients with incomplete $CD4^+$ gain.

We recently reported that the level of IP-10 in plasma was negatively associated with the $CD4^+$ cell count after 2.4 years in the individuals with profoundly impaired immune reconstitution at inclusion [16]. In the current study, we found more precisely that the IP-10 response in monocytes after exposure to HIV *in vitro* was negatively correlated with the $CD4^+$ gain after median 2.4 and 4.7 years both for the INR and immunological responder groups, with the strongest association in individuals with baseline $CD4^+$ cell count 300 cells/μl less. Type I interferons seem to be important for IP-10 expression in monocytes after HIV exposure [17], suggesting that pDCs could play a role in the IP-10 increase. IP-10 stimulates HIV-replication *in vitro* [53]. Moreover, in primary HIV-infection, IP-10 levels are strongly correlated with HIV RNA and cell-associated DNA and reported to be a better predictor of disease progression than the level of viremia [54,55]. In addition, IP-10 attracts $CXCR3^+CD4^+$ T cells which are major target cells for HIV and also contain the highest amount of integrated DNA in treated HIV infection [56]. Hence,

monocytes which respond to HIV with a high IP-10 production could be important in HIV pathogenesis and potentially unfavorable in the long term also in treated HIV infection.

Our study has some limitations. We have evaluated HIV-specific monocyte and dendritic cell responses, but inclusion of other stimuli such as LPS, flagellin, CpG-containing DNA and CMV, would have given a more comprehensive knowledge of factors contributing to monocyte and dendritic cell activation in INRs. Furthermore, dendritic cell subsets are rare cell populations and are sometimes too small for phenotyping and evaluation of IP-10 and IDO responses. However, studying PBMCs has an advantage over isolated cell populations as cytokine responses and in particular IP-10 is dependent of cross-talk between different immune cells [57]. Finally, about one third of the INRs experienced an increase in their $CD4^+$ cell count to more than 400 cells/μl after 4 years, in line with other studies [58]. Some of these patients were found in the groups selected for in-vitro AT-2 HIV assays. Although only INRs and immunological responders with similar age and nadir $CD4^+$ cell count were selected for this in-vitro assay, diverse potential for prospective $CD4^+$ recovery in the INR group could have contributed to the observed lack of differences in in-vitro responses between INRs and immunological responders. Thus, for future studies inclusion criteria with even longer duration of ART could be preferable.

In conclusion, ART-treated PLWH with an inadequate immune recovery had a more activated phenotype of monocytes and in particular dendritic cells, changes that were related to activation and regulation in the T-cell compartment. Of note, a high in-vitro HIV-induced IP-10 expression in monocytes was related to a lower future $CD4^+$ recovery. As type I interferons are important for IP-10 expression in monocytes after HIV exposure, an interplay between viral stimulation of pDCs, activation of monocytes and subsequent IP-10 expression could be a relevant pathway involved in inflammation and poor immune recovery during HIV infection.

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Authors' contributions: Conceived and designed the study: A.M.D.-R., B.S. Recruited the patients and collected clinical data: B.S. Performed the flow cytometry experiments: B.S., K.B.L. Performed the multiplex analyses: H.C.D.A. Performed the sCD163 ELISA analyses: T.U. Performed the statistical analyses: B.S. Analyzed the data: B.S., M.T., H.C.D.A., A.M.D.-R. Contributed reagents and analyses tools: B.S., A.M.D.-R., H.C.D.A., K.B.L., T.U. Drafted the article: B.S., A.M.D.-R. All authors have critically revised the article.

Conflicts of interest

There are no conflicts of interest.

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Supplementary methods

Soluble markers and T cell phenotypes

EDTA plasma and peripheral blood mononuclear cells (PBMC) were sampled at inclusion. Soluble markers of inflammation were analyzed in snap-frozen plasma by ELISA (IP-10, sCD14 and hsIL-6) and multiplex (IL-1β, IL-6, IL-7, IL-10, IL-22, Macrophage Chemoattractant Protein 1, MIP-1β and TNF), and LPS by Limulus Amebocyte Lysate colorimetric assay [1]. Plasma concentrations of tryptophan, kynurenine and neopterin were investigated by liquid chromatography-tandem mass spectrometry [2]. Cryopreserved PBMC were analyzed by 8-color flow cytometry and the fractions of the following $CD4^+$ and $CD8^+$ T cell subsets were determined; activated ($CD38^+HLA\text{-}DR^+$) and differentiated (naïve; $CD45RA^+CD27^+$, effector memory (EM); $CD45RO^+CD27^-$), and activated and resting regulatory T cells [aTregs; $\%CD147^{high}CD25^{high}$ of $CD4^+$ [3] and rTregs; $\%CD45RA^+Foxp3^+$ of $CD4^+$ (both gated from lymphocytes, singlets, $CD3^+CD4^+$ and then the respective Treg subset markers [4])]. More details and plots illustrating the gating strategy can be found in [4].

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TABLE S1. Antibodies and dyes used to determine monocyte and DC frequencies and activation and IP-10 and IDO upregulation after exposure to AT-2 HIV *in vitro*

	Flurochrome	Clone	Company	Location
Monocyte activation				
CD45	PB	HI30	BioLegend	San Diego, CA, USA
HLA-DR	APC-H7	G46-6	BD Biosciences	San Jose, CA, USA
Fixable Viability	eFluor 660		eBioscience, Thermo Fischer Scientific	Carlsbad, CA, USA
Lineage, anti CD3, CD19, CD20, CD56	APC	UCHT1, HIB19, 2H7, 5.1H11	BioLegend	San Diego, CA, USA
CD16	BV605	3G8	BD Biosciences	San Jose, CA, USA
CD14	Ax488	M5E2	BD Biosciences	San Jose, CA, USA
CD142	PE	HTF-1	BD Biosciences	San Jose, CA, USA
CD11b	PECy-7	ICRF44	BioLegend	San Diego, CA, USA
CD163	PerCp-Cy 5.5	GHI/61	BD Biosciences	San Jose, CA, USA
IgG1 κ isotype, mouse	PE		BD Biosciences	San Jose, CA, USA
IgG1 κ isotype, mouse	PECy-7		BioLegend	San Diego, CA, USA
IgG1 κ isotype, mouse	PerCp-Cy 5.5		BD Biosciences	San Jose, CA, USA
DC activation				
CD45	PB	HI30	BioLegend	San Diego, CA, USA
HLA-DR	APC-H7	G46-6	BD Biosciences	San Jose, CA, USA
Fixable Viability	eFluor 520		eBioscience, Thermo Fischer Scientific	Carlsbad, CA, USA
Lineage 3, anti CD3, CD14, CD19, CD20,	FITC	SK7, MϕP9, SJ25C1, L27	BD Biosciences	San Jose, CA, USA
CD1c	PE-Vio770	AD5-8E7	Miltenyi Biotec GmbH	Bergisch Gladbach, Germany
CD141	APC	AD5-14H12	Miltenyi Biotec GmbH	Bergisch Gladbach, Germany
CD303	BV605	201A	BioLegend	San Diego, CA, USA
CD83	PE	HB15e	BioLegend	San Diego, CA, USA

CD86	PerCp eFluor 710	IT2.2	eBioscience, Thermo Fischer Scientific	Carlsbad, CA, USA
IgG2b κ isotype, mouse	PerCp eFluor 710		eBioscience, Thermo Fischer Scientific	Carlsbad, CA, USA
Tandem Signal Enhancer			Miltenyi Biotec GmbH	Bergisch Gladbach, Germany
IDO and IP-10 upregulation in monocytes after exposure to AT-2 HIV *in vitro*				
CD45	PB	HI30	BioLegend	San Diego, CA, USA
HLA-DR	APC-H7	G46-6	BD Biosciences	San Jose, CA, USA
Fixable Viability	eFluor 660		eBioscience, Thermo Fischer Scientific	Carlsbad, CA, USA
Lineage, anti CD3, CD19, CD20, CD56	APC	UCHT1, HIB19, 2H7, 5.1H11	BioLegend	San Diego, CA, USA
CD16	BV605	3G8	BioLegend	San Diego, CA, USA
CD14	Ax488	M5E2	BD Biosciences	San Jose, CA, USA
IP-10	PE	6D4/D6/G2	BD Biosciences	San Jose, CA, USA
IDO	PerCp-eFluor 710	eyedio	eBioscience, Thermo Fischer Scientific	Carlsbad, CA, USA
IDO and IP-10 upregulation in DC after exposure to AT-2 HIV *in vitro*				
CD45	PB	HI30	BioLegend	San Diego, CA, USA
HLA-DR	APC-H7	G46-6	BD Biosciences	San Jose, CA, USA
Fixable Viability	eFluor 520		eBioscience, Thermo Fischer Scientific	Carlsbad, CA, USA
Lineage 3, anti CD3, CD14, CD19, CD20,	FITC	SK7, MϕP9, SJ25C1, L27	BD Biosciences	San Jose, CA, USA
CD1c	PE-Vio770	AD5-8E7	Miltenyi Biotec GmbH	Bergisch Gladbach, Germany
CD141	APC	AD5-14H12	Miltenyi Biotec GmbH	Bergisch Gladbach, Germany
CD303	BV605	201A	BioLegend	San Diego, CA, USA

IP-10	PE	6D4/D6/G2	BD Biosciences	San Jose, CA, USA
IDO	PerCp-eFluor 710	eyedio	eBioscience, Thermo Fischer Scientific	Carlsbad, CA, USA
Tandem Signal Enhancer			Miltenyi Biotec GmbH	Bergisch Gladbach, Germany

DC, dendritic cells; AT-2 HIV, aldrithiol-2 inactivated HIV-1

TABLE S2. Characteristics of the Subgroups for *in vitro* AT-2 HIV stimulation at Inclusion

Total study population	**INR (n=20)**	**IR (n=20)**	**HC (n=8)**	**P-value**[a]
Age, (IQR)	47.4 (41.0-56.5)	45 (40.2-55.3)	46.4 (40.4-57.3)	NS
Male gender, n (%)	18 (90)	15 (75)	6 (75)	NS
Ethnicity, n (%)				
Caucasian	14 (70)	13 (65)	8 (100)	NS
Risk group, n (%)				
MSM	9 (45)	10 (50)		NS
Other[1]	11 (55)	10 (50)		NS
Comorbid diseases, n (%)				
Cardiovascular	2 (10)	0 (0)		NS
Any comorbidity[2]	9 (45)	5 (25)		NS
CMV IgG pos	20 (100)	20 (100)		NS
HIV characteristics, (IQR)				
Years since HIV diagnosis	8.1 (5.4-13.5)	9.5 (7.5-14.3)		NS
Years of continuous ART	5.5 (3.5-6.1)	6.7 (5.9-8.6)		**0.01**
Viral load at ART initiation, cop/mL	41000 (25600-71000)	160000 (65400-460000)		**0.02**
Duration of viral suppression, years	3.8 (2.6-5.5)	6.2 (4.4-7.6)		**0.01**
Viral load at inclusion, cop/mL	≤20	≤20		NS
CD4 count nadir, cells/µL	151.5 (95-165)	148.5 (94-190)		NS
CD4 count at inclusion, cells/µL	309.5 (242.5-358)	795.5 (732-847.5)		**<0.001**
CD8 count at inclusion, cells/µL	665.5 (544-921)	989.5 (796-1575.5)		**0.002**
CD4/CD8 at inclusion	0.44 (0.35-0.55)	0.79 (0.55-0.99)		**<0.001**

Data are presented as no. (%) of study participants or median (interquartile range (IQR)) values.

[a]P-values for Kruskal-Wallis Test or Pearson Chi-Square test for comparison between multiple groups, Mann-Whitney U Test or Fischer's Exact Test for comparison INR vs IR.

[1]Other. Heterosexual or unknown. There were no intravenous drug abusers.

[2]One or more of the following comorbidities; cardiovascular disease, hypertension, diabetes, renal disease, osteoporosis, chronic obstructive pulmonary disease, neurodegenerative disease, previous cancer or Mycobacterium tuberculosis infection.

AT-2 HIV, aldrithiol-2 inactivated HIV-1; INR, immunological non-responders; IR, immunological responders; HC, healthy control; MSM, men who have sex with men; CMV, cytomegalovirus; ART, antiretroviral therapy.

TABLE S3. INR group (n=38): Correlation between HIV baseline characteristics and CD4 count and CD4/CD8 after four years and the increase in CD4 and CD4/CD8 from inclusion

	CD4 (cells/µL) (rho)	**CD4 increase (rho)**	**CD4/CD8 (rho)**	**CD4/CD8 increase (rho)**
Duration of continuous ART	**-0.40***	**-0.55*****	**-0.63*****	**-0.47****
Duration of continuous viral suppression	-0.26	**-0.37***	**-0.48****	**-0.42***
Duration of HIV infection	**-0.33***	**-0.33***	**-0.46****	**-0.35***
Nadir CD4	**0.46****	**0.35***	-0.31	0.15
Age	**-0.38***	-0.32	**-0.37***	-0.25

Spearman rank order correlation. * P < 0.05, ** P < 0.01, *** P < 0.001

INR, immunological non-responder; ART, antiretroviral therapy

TABLE S4. Correlation between expression of activation markers on monocytes or DC subsets and T cell activation and differentiation, aTregs/rTregs, and CD4 count at inclusion, in INR and IR

Cell population		Act. $CD4^+$ T cells (rho)	Act. $CD8^+$ T cells (rho)	EM $CD4^+$ T cells (rho)	Naïve/ EM $CD8^+$ T cells (rho)	aTregs/ rTregs (rho)	CD4 count (cells/µL) (rho)
Total monocytes	**MFI HLA-DR**	**0.62****	**0.48***	**0.42****	**-0.37***	0.05	**-0.32****
$CD141^{++}$ mDC	**$\%CD83^+$**	0.43	0.47	0.18	-0.20	0.16	**-0.42****
	$\%CD86^+$	**0.57***	**0.51***	0.30	-0.34	**0.40***	**-0.54*****
	MFI CD86	0.38	0.12	0.20	-0.18	0.31	**-0.45*****
	$\%CD83^+CD86^+$	0.43	**0.51***	0.20	-0.28	0.25	**-0.49*****
$CD1c^+$ mDC	**MFI HLA-DR**	**0.62****	0.38	**0.33***	**-0.33***	0.09	**-0.36****
	MFI CD86	**0.44***	0.27	0.27	-0.23	**0.31***	**-0.56*****
$CD303^+$ pDC	**$\%CD83^+$**	**0.60****	**0.47***	0.19	-0.28	**0.31***	**-0.43****
	MFI CD83	**0.65****	**0.52***	0.18	-0.24	**0.40****	**-0.37****

Spearman rank order correlation. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

DC, dendritic cells; mDC, myeloid dendritic cells; pDC, plasmacytoid dendritic cells; Act, activated cells ($CD38^+HLA\text{-}DR^+$); EM, effector memory cells ($CD45RO^+CD27^-$); naïve ($CD45RA^+CD27^+$), aTregs; activated regulatory T cells ($\%CD147^{high}CD25^{high}$ of $CD4^+$); rTregs, resting Tregs ($\%CD45RA^+Foxp3^+$ of $CD4^+$); MFI, median fluorescence intensity.

TABLE S5. Correlation between cytokines responses in supernatants and IP-10 and IDO expression in monocytes and mDC after exposure to AT-2 HIV *in vitro* in INR and IR

	INR (n=20)				**IR (n=20)**
	Monocytes			**mDC**	**mDC**
Supernatants (pg/mL)	**MFI IP-10 (rho)**	**% IP-10 (rho)**	**MFI IDO (rho)**	**MFI IP-10 (rho)**	**MFI IP-10 (rho)**
MIP-1β	**0.45***	0.35	**0.53***	**0.56***	**0.55***
IFNα2	**0.63****	0.37	0.28	**0.58****	-0.22
IL-1ra	**0.59****	**0.45***	0.14	**0.65****	0.26
IL-18	**0.63****	0.17	0.35	**0.47***	-0.03
IL-1β	0.41	-0.12	0.04	0.06	-0.11
IFNγ	0.41	0.15	0.43	**0.47***	-0.04
TNFα	0.39	0.14	0.31	0.42	0.01
IP-10	**0.67****	**0.53***	0.20	**0.63****	0.44
IL-6	0.30	-0.16	0.12	0.08	-0.13

Correlation between delta values (mock stimulation subtracted).

Spearman rank order correlation. * $P < 0.05$, ** $P < 0.01$

IDO, indoleamine 2, 3 dioxygenase; mDC, myeloid dendritic cells; AT-2 HIV, aldrithiol-2-inactivated HIV-1; INR, immunological non-responder; IR, immune responder; MFI, median fluorescence intensity.

Figure S1

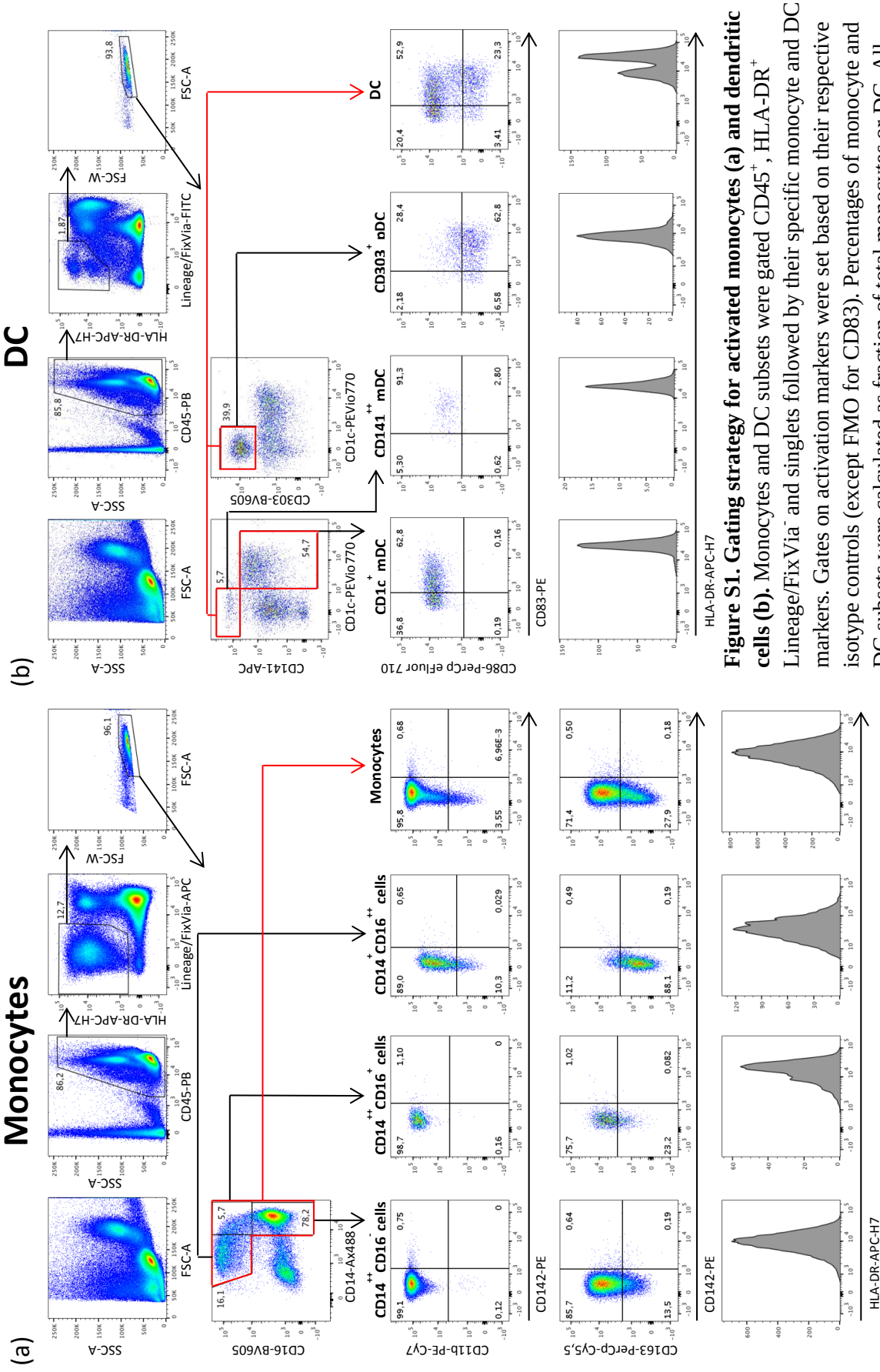


Figure S1. Gating strategy for activated monocytes (a) and dendritic cells (b). Monocytes and DC subsets were gated $CD45^+$, $HLA\text{-}DR^+$ $Lineage/FixVia^-$ and singlets followed by their specific monocyte and DC markers. Gates on activation markers were set based on their respective isotype controls (except FMO for CD83). Percentages of monocyte and DC subsets were calculated as fraction of total monocytes or DC. All figures are from one representative HIV immunological non-responder patient. DC, dendritic cells. Lineage, monocyte panel: anti-CD3, CD19, CD20, CD56. Lineage, DC panel; anti-CD3, CD14, CD19, CD20. FixVia, fixable viability stain.

Figure S2

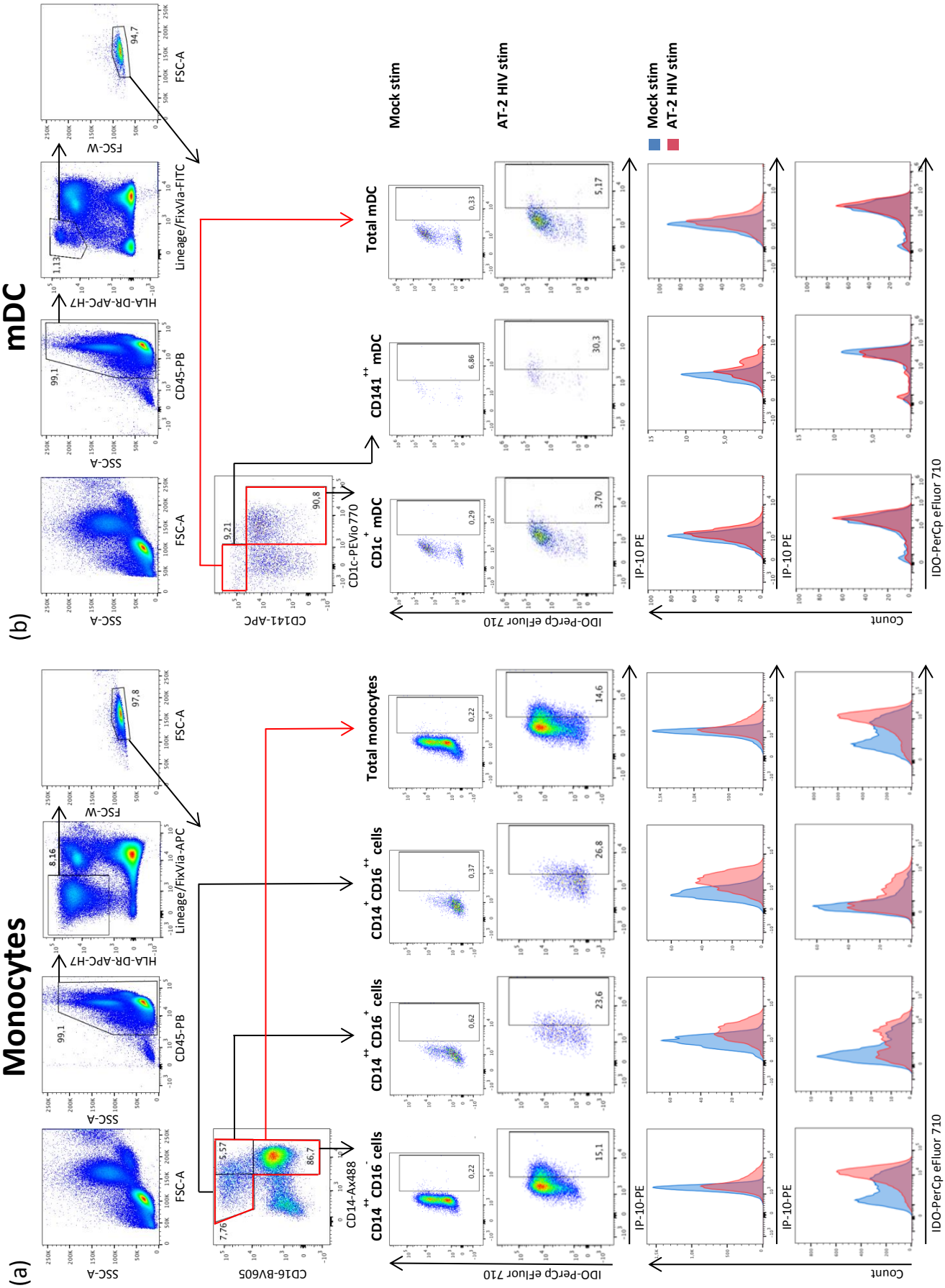

(a)
Monocytes
SSC-A
FSC-A
CD45-PB
HLA-DR-APC-H7
Lineage/FixVia-APC
FSC-W
FSC-A
CD16-BV605
CD14-Ax488
CD14++ CD16- cells
CD14++ CD16+ cells
CD14+ CD16++ cells
Total monocytes
IDO-PerCp eFluor 710
IP-10-PE
Count
(b)
mDC
SSC-A
FSC-A
CD45-PB
HLA-DR-APC-H7
Lineage/FixVia-FITC
FSC-W
FSC-A
CD141-APC
CD1c-PEVio770
CD1c+ mDC
CD141++ mDC
Total mDC
Mock stim
AT-2 HIV stim
IDO-PerCp eFluor 710
IP-10 PE
Count

Figure S2. Gating strategy for IP-10 and IDO expression in monocytes (a) and mDC (b) after exposure to mock and AT-2 HIV *in vitro*. Monocytes and mDC subsets were gated from $CD45^{+}$, $HLA\text{-}DR^{+}$ Lineage/$FixVia^{-}$ and singlets followed by IP-10 and IDO expression. Due to reduction of the CD303 expression in this assay, IP-10 and IDO upregulation were not analyzed in pDC. Gates on IP-10 and IDO were based on mock stimulated sample as negative control. Percentages of monocyte and mDC subsets were calculated as fraction of total monocytes or mDC. The histograms display overlay plot of mock and AT-2 HIV stimulated samples for IP-10 and IDO, respectively. All figures are from one representative HIV immunological non-responder patient. mDC, myeloid dendritic cells; AT-2 HIV, aldrithiol-2 inactivated HIV-1. Lineage, monocyte panel: anti-CD3, CD19, CD20, CD56. Lineage, DC panel; anti-CD3, CD14, CD19, CD20. FixVia, fixable viability stain.

Paper III

Figure S3

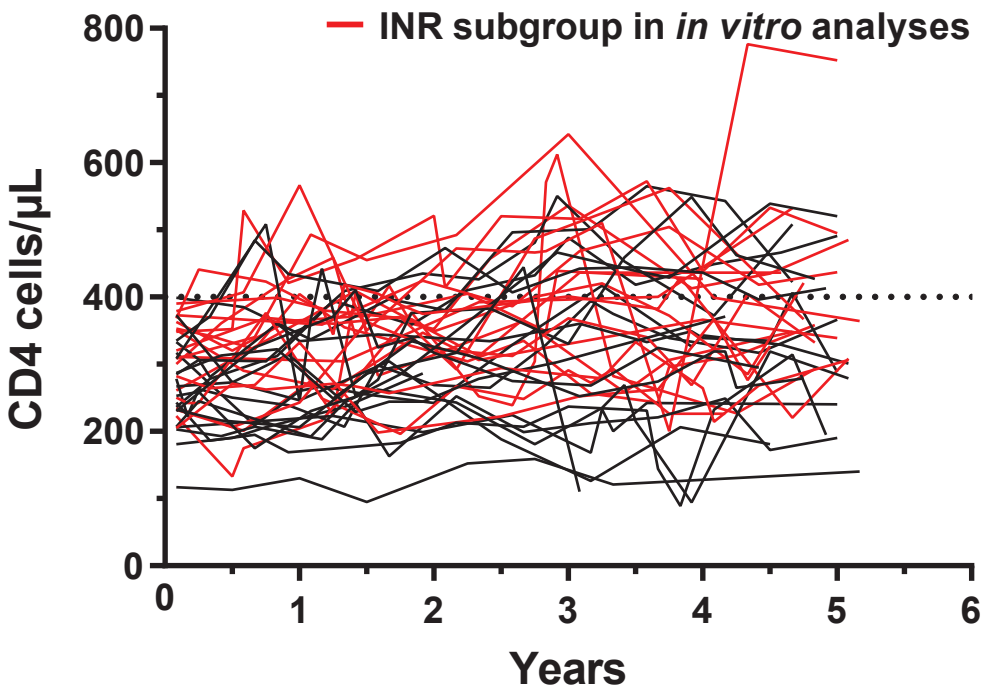


Figure S3. Line-plots showing CD4 counts in INR from inclusion and median 4.7 years prospectively.

Line-plots showing CD4 counts in the INR cohort obtained at inclusion and from all routine clinical visits prospectively for median 4.7 years. INR patients included in *in vitro* AT-2 HIV stimulation assays are shown in red. INR, immunological non-responder; AT-2 HIV, aldrithiol-2 inactivated HIV-1 virus.

Figure S4

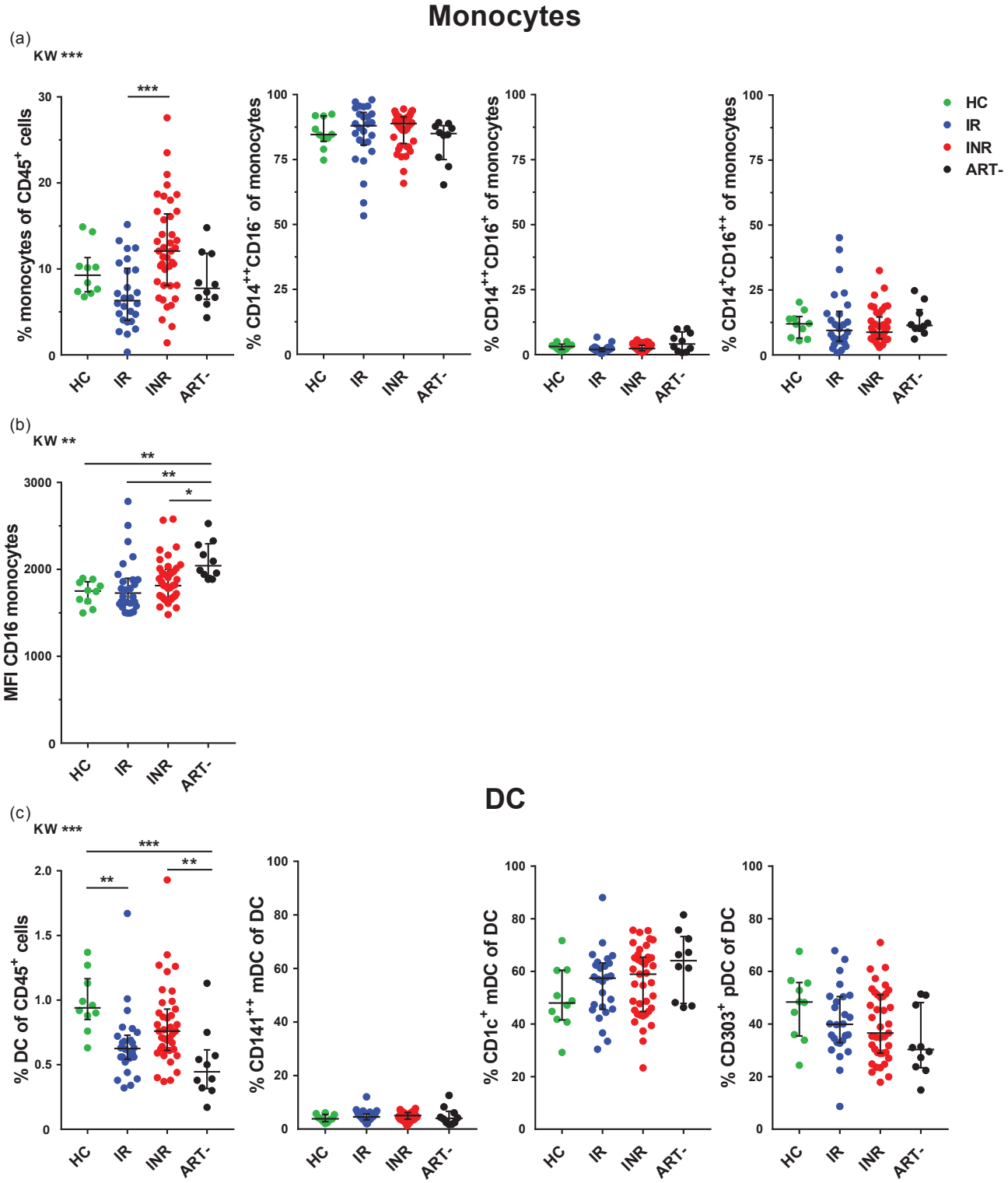
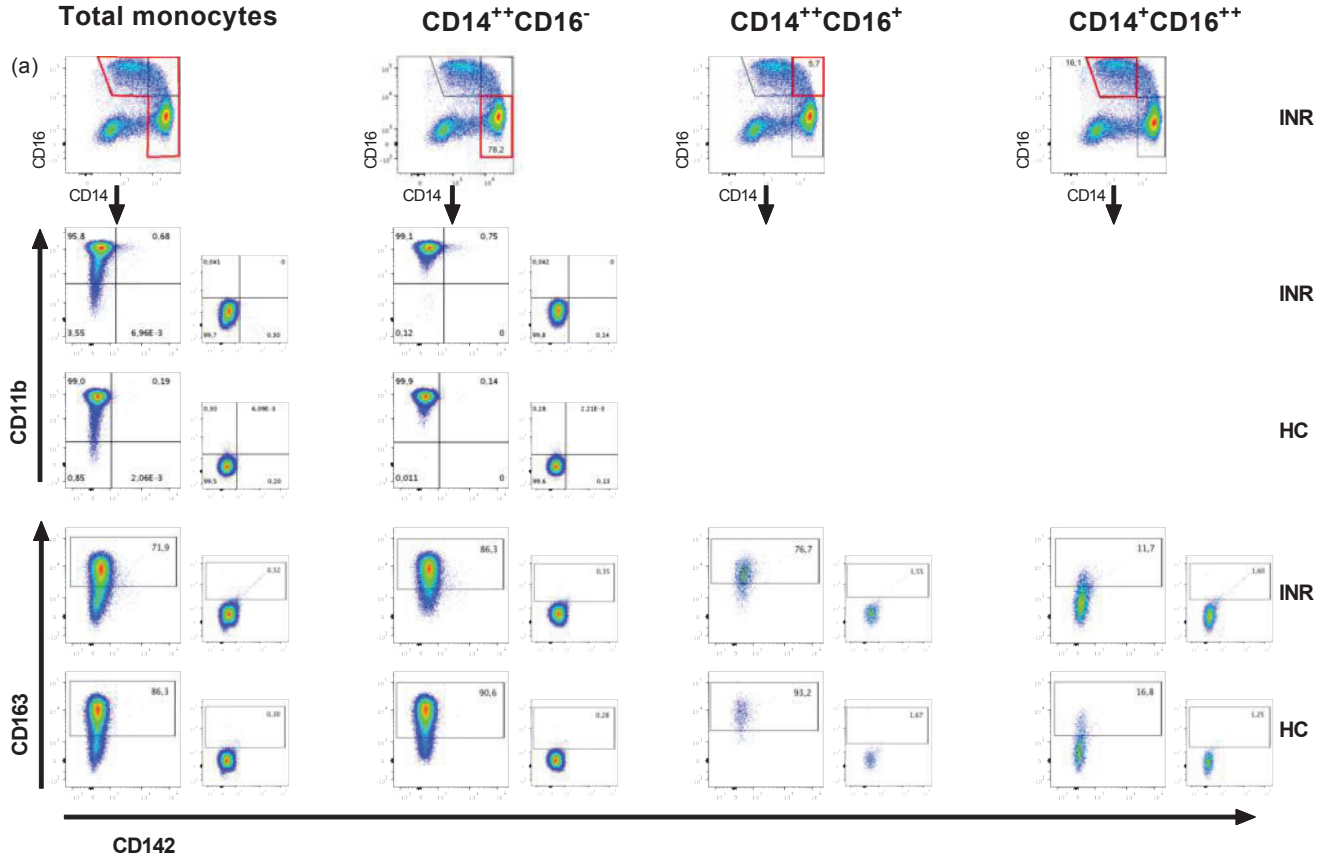
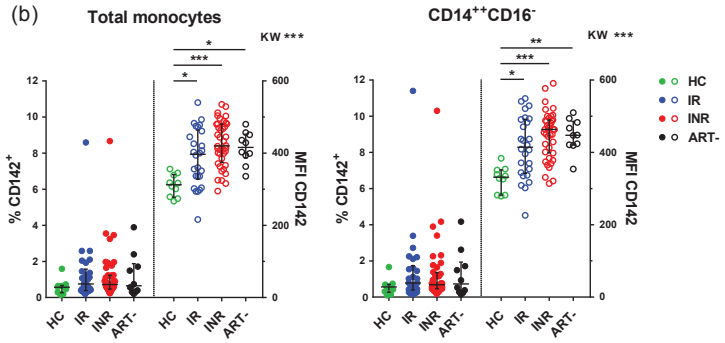
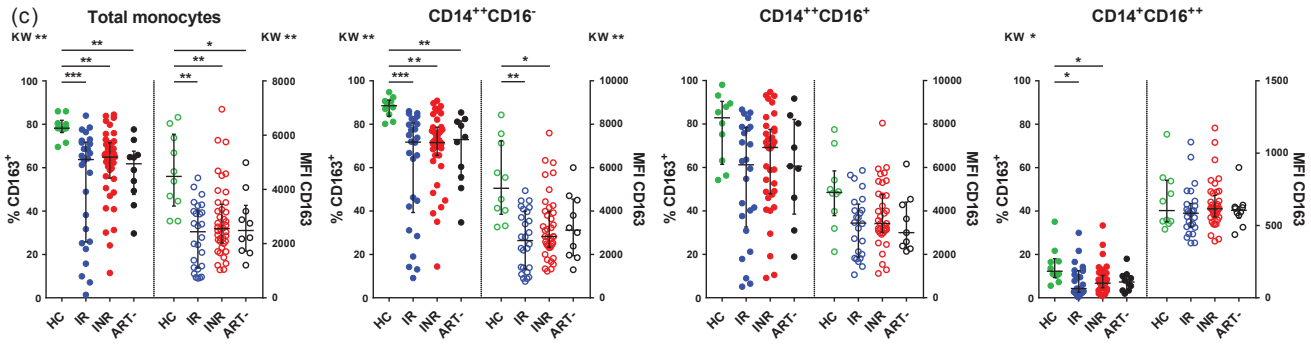


Figure S4. Distribution of monocytes and dendritic cell subsets in different study groups. (a) displays percentages of total monocytes of CD45 $^{+}$ cells and the distribution of specific monocyte subsets in the different study groups and (c) the same for total DC and DC subsets. In (b) MFI values of CD16 in monocytes are shown. Kruskal-Wallis Test (KW) followed by Dunn's posthoc test. * P < 0.05, ** P < 0.01, *** P < 0.001. Lines indicate median and interquartile range. DC, dendritic cells; MFI, median fluorescence intensity; HC, healthy control; IR, immune responder; INR, immunological non-responder; ART-, antiretroviral therapy naive.

Figure S5


Total monocytes
CD14++CD16-
CD14++CD16+
CD14+CD16++
(a)
CD16
CD14
CD11b
CD163
CD142
INR
INR
HC
INR
HC


(b)
Total monocytes
CD14++CD16-
KW ***
% CD142+
MFI CD142
HC
IR
INR
ART-


(c)
Total monocytes
CD14++CD16-
CD14++CD16+
CD14+CD16++
KW **
KW *
% CD163+
MFI CD163
HC
IR
INR
ART-

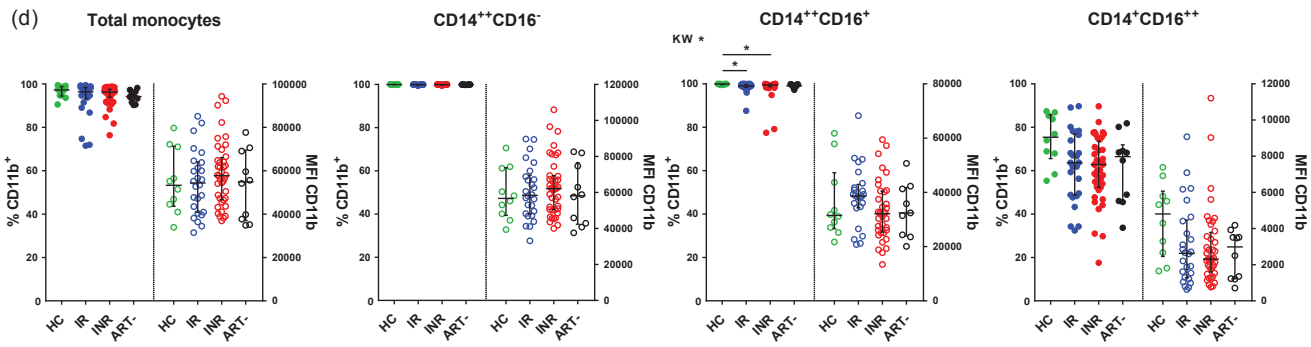

(d)
Total monocytes
CD14++CD16-
CD14++CD16+
CD14+CD16++
KW *
% CD11b+
MFI CD11b
HC
IR
INR
ART-

Figure S5. Monocyte activation markers in the different cohorts. (a) illustrate gating strategy of monocyte activation markers based on isotype as negative control in total monocytes and different monocyte subsets. The plots show representative examples from one INR and one HC. In (b), (c), and (d) the graphs display both percentages (solid dots) and MFI (open dots) values of the activation markers CD142, CD163 and CD11b in the specific monocyte subsets for each group. Kruskal-Wallis Test (KW) followed by Dunn's posthoc test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Lines indicate median and interquartile range. INR, immunological non-responder; HC, healthy control; IR, immune responder; ART-, antiretroviral therapy naïve HIV-infected; MFI, Median fluorescence intensity.

Paper III

Figure S6

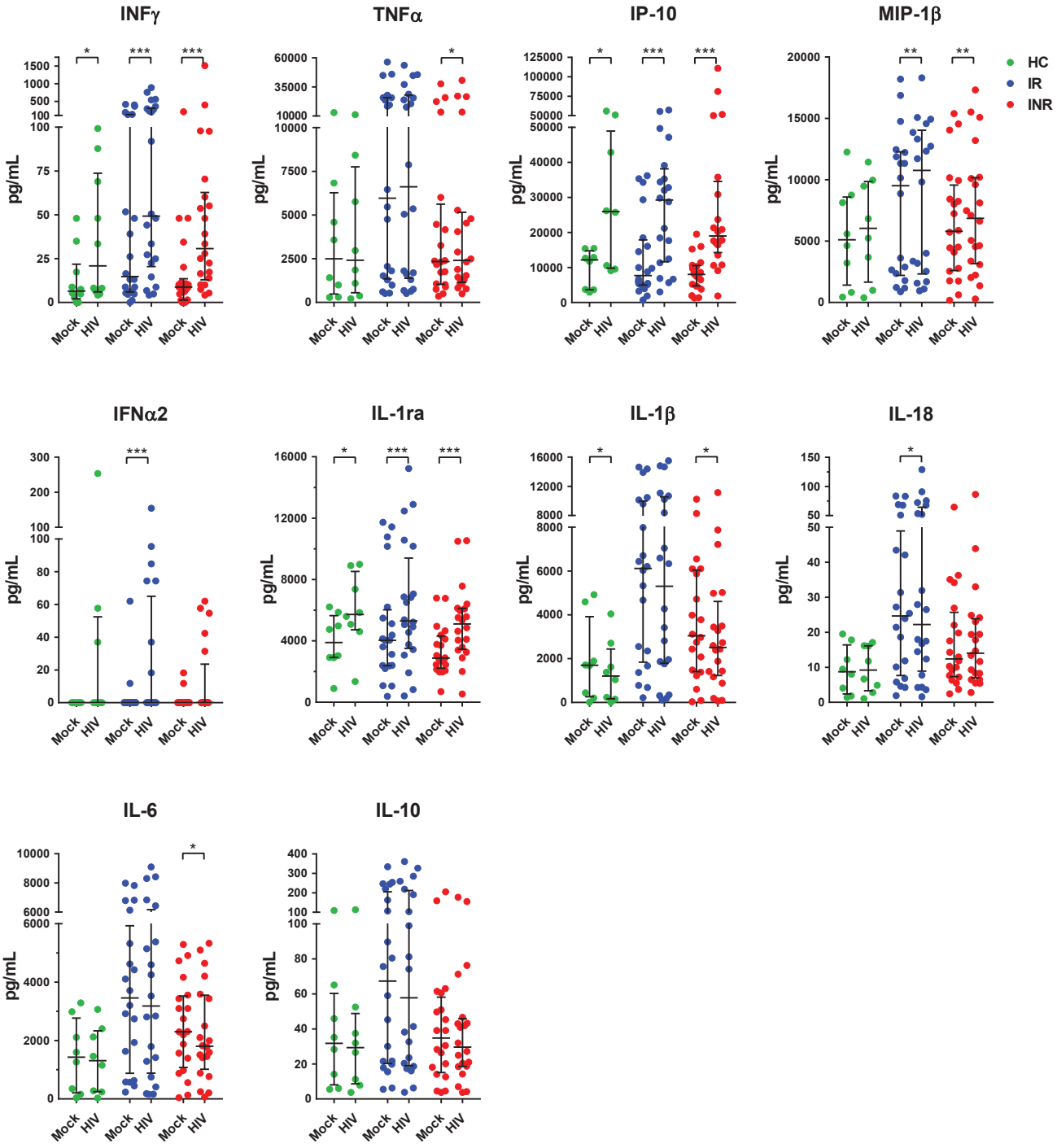


Figure S6. Cytokine responses in supernatants after exposure to AT-2 HIV *in vitro*.

This figure shows various cytokine responses in supernatants after *in vitro* stimulation with either mock or AT-2 HIV in the HC, IR and INR subgroups. There were no significant differences in the cytokine responses between the groups (Kruskal-Wallis Test). Wilcoxon Test for pairwise comparisons between mock and AT-2 HIV stimulation. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Lines indicate median and interquartile range. AT-2 HIV, aldrithiol-2 inactivated HIV-1 virus; HC, healthy control; IR, immune responder; INR, immunological non-responder.

Erratum

Page	Line	Original text	Corrected text
x	last	AIDS. 2019;33:1117-11293	AIDS. 2019;33:1117-1129
1 2	last 1	Subtype B is the most prevalent worldwide and predominates in Africa and India, whereas subtype C predominates in Europe,…	Subtype C is the most prevalent worldwide and predominates in Africa and India, whereas subtype B predominates in Europe,…
9	19	...regardless of WHO clinical stage and at any CD4 cell count, in 2016 [38].	...regardless of WHO clinical stage and at any CD4 cell count, in 2015 [38].
10	5	…or at least at "functional" HIV cure…	…or at least a "functional" HIV cure…
10	11	…although "the Berlin patients"…	…although "the Berlin patient"…
11	2	… contribute to a partial over the infection,…	…contribute to a partial control over the infection,…
13	9	…in virally suppressed PLWP..	…in virally suppressed PLWH..
24	27	… *Lacobacillus* species…	… *Lactobacillus* species…
28	2	…address this question in ART-treated P…	…address this question in ART-treated PLWH…
34	Fig. 12 legend	…Expected age at death of women (right panel) and men (left panel)…	…Expected age at death of women (left panel) and men (right panel)…
49	Table II	CD4 count also registered 2 years after inclusion CD4 count also registered 2 and 4 years after inclusion HIV RNA < 50 copies/mL HIV RNA ≤ 20 copies/mL Duration of HIV infection ≥ 1 year	[a]CD4 count also registered 2 years after inclusion [b]CD4 count also registered 2 and 4 years after inclusion [c]HIV RNA < 50 copies/mL [d]HIV RNA ≤ 20 copies/mL [e]Duration of HIV infection ≥ 1 year
94	1	In 2016, updated WHO guidelines recommended lifelong ART…	In 2015, updated WHO guidelines recommended lifelong ART…
98	Ref. 38	38. Organization WH. Consolidated guidelines on the use of antiretroviral drugs for treating and preventing HIV infection. Recommendations for a public health approach - Second edition. https://www.who.int/hiv/pub/arv/arv-2016/en/: World Health Organization, 2016.	38. Organization WHO. Consolidated guidelines on the use of antiretroviral drugs for treating and preventing HIV infection: what's new. https://www.who.int/hiv/pub/arv/policy-brief-arv-2015/en/: World Health Organization, 2015.

Appendix

Table I. Antibodies and dyes used in T cell phenotyping assays in paper I + II.

Marker	Fluorochrome	Laser	Filter	Clone
T cell phenotyping, paper I (BD FACSCanto II)				
CD3	V450	Violet 405	450/50	UCHT1
HLA-DR	BV605	Violet 405	616/23	G46-6
CD161	APC	Red 633	660/20	DX12
CD8	APC-H7	Red 633	780/60	SK1
CD38	PE	Blue 488	585/42	HB7
CD127	PE-Cy7	Blue 488	780/60	HIL-7R-M21
CD25	PerCp-Cy 5.5	Blue 488	695/40	M-A251
CD45RA	FITC	Blue 488	530/30	HI100
T cell differentiation and activation, paper II (BD LSR Fortessa)				
CD3	Pacific Blue	Violet 405	450/50	UCHT1
HLA-DR	BV605	Violet 405	605/12	G46-6
CD279 (PD-1)	APC	Red 640	670/30	MIH4
CD8	APC-H7	Red 640	780/60	SK1
CD38	PE	Blue 488	575/26	HB7
CD27	PE-Cy7	Blue 488	780/60	M-T271
CD45RO	PerCp-Cy 5.5	Blue 488	695/40	UCHL1
CD45RA	FITC	Blue 488	530/30	HI100
Tregs, paper II (BD LSR Fortessa)				
CD3	Pacific Blue	Violet 405	450/50	UCHT1
CD161	BV605	Violet 405	605/12	DX12
FoxP3	Ax647 (APC)	Red 640	670/30	259D/C7
CD45RA	APC-H7	Red 640	780/60	HI100
CD25	PE	Blue 488	575/26	4E3
CD127	PE-Cy7	Blue 488	780/60	eBioRDR5
$CD4^+$ T cells	PerCp	Blue 488	695/40	L200
CD147	FITC	Blue 488	530/30	HIM6

Table II. Antibodies and dyes used in monocyte and DC assays in paper III.

Marker	Fluorochrome	Laser	Filter	Clone	Isotype
Monocyte phenotyping (BD FACSCanto II)					
CD45	Pacific Blue	Violet	450/50	H130	
CD16	BV605	Violet	616/23	3G8	
Lineage (anti-CD3, CD19, CD20, CD56)	APC	Red 633	660/20	UCHT1, HIB19, 2H7,	
Fixable Viability	eFluor 660 (APC)	Red 633	660/20		
HLA-DR	APC-H7	Red 633	780/60	G46-6	
CD142	PE	Blue 488	585/42	HTF-1	IgG1 κ
CD11b	PE-Cy7	Blue 488	780/60	ICRF-44	IgG1 κ
CD163	PerCp-Cy 5,5	Blue 488	695/40	GHI/61	IgG1 κ
CD14	Ax488	Blue 488	530/30	M5E2	
Monocyte stimulation (BD FACSCanto II)					
CD45	Pacific Blue	Violet	450/50	H130	
CD16	BV605	Violet	616/23	3G8	
Lineage (anti-CD3, CD19, CD20, CD56)	APC	Red 633	660/20	UCHT1, HIB19, 2H7,	
Fixable Viability	eFluor 660 (APC)	Red 633	660/20		
HLA-DR	APC-H7	Red 633	780/60	G46-6	
IP-10	PE	Blue 488	585/42	6D4/D6/G2	
IDO	PerCp eFluor 710	Blue 488	695/40	eyedio	
CD14	Ax488	Blue 488	530/30	M5E2	
DC phenotyping (BD FACSCanto II)					
CD45	Pasific Blue	Violet	450/50	H130	
CD303	BV605	Violet	616/23	201A	
CD141	APC	Red 633	620/20	AD5-14H12	
HLA-DR	APC-H7	Red 633	780/60	G46-6	
CD83	PE	Blue 488	585/42	HB15e	
CD1c	PE-ViO770	Blue 488	780/60	AD5-8E7	
CD86	PerCp eFluor 710	Blue 488	695/40	IT2.2	IgG2b κ
Lineage 3 (anti-CD3, CD14, CD19, CD20)	FITC	Blue 488	530/30	SK7, MϕP9, SJ25C1, L27	
Fixable Viability	eFluor 520	Blue 488	530/30		

Table II. Cont.

Marker	Fluorochrome	Laser	Filter	Clone	Isotype
DC stimulation (BD FACSCanto II)					
CD45	Pasific Blue	Violet	450/50	H130	
CD303	BV605	Violet	616/23	201A	
CD141	APC	Red 633	620/20	AD5-14H12	
HLA-DR	APC-H7	Red 633	780/60	G46-6	
IP-10	PE	Blue 488	585/42	6D4/D6/G2	
CD1c	PE-ViO770	Blue 488	780/60	AD5-8E7	
IDO	PerCp eFluor 710	Blue 488	695/40	eyedio	
Lineage 3 (anti-CD3, CD14, CD19, CD20)	FITC	Blue 488	530/30	SK7, MϕP9, SJ25C1, L27	
Fixable Viability	eFluor 520	Blue 488	530/30		