Immune exhaustion of T cells in HIV+ immunological nonresponders

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Charlotte Andrea Hauge Handeland

Abstract

The treatment for HIV infected patients has been greatly improved over the last decades. Antiretroviral treatment (ART) prevents the development of AIDS and suppresses the virus. Still, some patients do not increase the CD4+ T cell numbers to normal levels. This group of patients has an increased risk of cardiovascular disease, cancer and immune failure, as well as death. We wanted to study if these so-called immunological non-responders display a more exhausted T cell phenotype compared to immunological responders and HIV negative persons. By utilizing flow cytometry, the expression of several cell surface markers typical for activation, exhaustion, and senescence have been analysed. We found that immunological nonresponders have increased percentages of T cells expressing PD-1 and TIGIT compared to patients who have a normal recovery of CD4+ T cells after treatment and HIV negative persons. HIV infection also compromises the integrity of the gut mucosa leading to increased immune activation, microbial translocation and systemic inflammation. Immunological nonresponders also showed upregulation of PD-1 and TIGIT on gut-homing CD8+ T cells.

This Thesis also provides a functional *in vitro* T cell stimulation assay that can be used to block PD-1 and TIGIT pathways with monoclonal antibodies where the aims are to reverse this exhausted T cell phenotype in immunological nonresponders into well-functional T cells capable of reconstituting the CD4+ T cell pool to normal levels.

Abbreviations

Abs	Antibodies
AIDS	Acquired immunodeficiency syndrome
APC	Antigen specific cell
ART	Antiretroviral treatment
CCR5	C-C chemokine receptor type 5
CD	Cluster of differentiation
cDNA	Complementary DNA
CM	Central memory
CTLA-4	Cytotoxic T-lymphocyte antigen 4
CTL	Cytotoxic lymphocyte
CXCR4	C-X-C chemokine receptor 4
DC	Dendritic cell
e.g	Exempli gratia
EM	Effector memory
Env	Envelope
FCS	Fetal calf serum
FMO	Fluorescence minus one
FSC	Forward side scatter
Gag	Group-specific antigen
GALT	Gut-associated lymphoid tissue
Gp	Glycoprotein
HC	Healthy control
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen

IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
INR	Immunological nonresponder
IR	Immunological responder
ITAM	Immunoreceptor tyrosine-based activation motif
ITG	Integrin
KLRG1	Killer-cell lectin receptor G1
Lag-3	Lymphocyte-activation gene 3
mAbs	Monoclonal antibodies
MHC	Major histocompatibility complex
NK	Natural killer
NKT	Natural killer T cell
NRTI	Nucleoside reverse transcriptase inhibitor
NNRTI	Non-nucleoside reverse transcriptase inhibitor
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate – buffered saline
PD-1	Programmed cell death protein 1
PD-L	Programmed death ligand
PI	Protease inhibitor
SSC	Side scatter
TCR	T cell receptor
TemRA	Terminally differentiated effector memory cell
Th	T helper cell
Tim-3	T-cell immunoglobulin and mucin-domain containing 3
TNF	Tumor necrosis factor

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1 Introduction

1.1 The immune system

The immune system is an organism's natural defense against foreign agents such as bacteria, virus, fungi and parasites. Other substances that appear foreign and harmful like cancerous cells or nonliving substances such as toxins, chemicals, drugs and foreign particles can also be reacted upon. Antigens are parts of proteins which the immune system can react upon and where it would try to destroy the organism that presents it [1]. The skin and mucosal surfaces such as the respiratory, gastrointestinal and urogenital tracts, forms the outer barrier of the body. If this barrier is disrupted, the innate immune system is activated. The innate immune system is present at birth and activation leads to a fast, unspecific response with no long-term memory. The response is mediated by molecular mechanisms such as the complement system and cellular responses by phagocytic macrophages and natural killer cells (NK cells) that try to eliminate foreign agents. If the innate immune system fails to defeat the threats, the adaptive immune system adds to the ongoing immune response. Dendritic cells (DCs) and macrophages phagocytose foreign agents and present this as short peptides on their cell surface by molecules called major histocompatibility complex (MHC class) I or II. The peptides on the MHC molecules are presented to T lymphocytes, which under the right circumstances will get activated and start to proliferate to create a clone with the same antigen specificity. The T cells differentiate into cells with effector functions and some become memory cells that can provide a long-term immunity against recurring agents [2]. T cells carry out cell-mediated immune responses through the production of cytokines and cytotoxic activity of effector T cells. Activated T cells also support B lymphocyte maturation and cell growth. B cells respond to pathogens through the B cell receptor. After activation, the B cell will secrete antibodies (Abs) that can bind to epitopes on the specific pathogens leading to opsonization of pathogen for engulfment by phagocytes [1]. Different aspects of T cell exhaustion have been studied in this Master Thesis and T cells will therefore be the focus in the rest of the Thesis.

1.1.1 T cell development

T cell progenitors originate from hematopoietic stem cells in the bone marrow but enter the thymus to differentiate and undergo selection and maturation into functional T cells. In the first steps of T cell maturation, the T cells are double negative cells which express neither the CD4 nor the CD8 co-receptor. These double negative T cells can give rise to either αβ or γδ T cell receptors (TCRs). To create the enormous clonal diversity of TCRs, T cells must undergo rearrangement within the α and β encoding genes and γ and δ encoding genes to generate the highly specific antigen-binding site. Depending on which lineage produces a productive TCR first, the T cell becomes committed to express an αβ or a γδ TCR [3]. The majority of T cells expresses the TCR as a αβ heterodimer and this will therefore be the focus for the rest of this Thesis. Successful rearrangement leads to expression of a potential TCR and the cells becomes double positive by expressing both CD4 and CD8 co-receptors. The TCR will associate with the CD3 complex consisting of the invariant polypeptides CD3ε, CD3γ, CD3δ and CD3 ζ chain [4]. The signaling complex is necessary for the transduction of signals to the interior of the cell when the TCR has bound an antigen, as the TCR itself has very short intracellular domains. Double positive T cells with a potential functional TCR will undergo positive selection. It is essential that TCRs can bind to the individual's own MHC molecules in the recognition of pathogen-derived peptides. T cells that bind to own MHC molecules are positively selected and mature further and differentiate into either CD4 or CD8 single positive cells. Single positive T cells that react and bind to self-antigens will undergo negative selection [5, 6]. The small fraction of naive CD4+ and CD8+ T cells that remains will exit the thymus and migrate between the blood, lymphatic system, and peripheral lymphoid organs in response to chemokines in a constant search for antigens [7].

1.1.2 Activation of T cells

CD4+ and CD8+ T cells recognize peptides presented on MHC molecules. CD4+ T cells recognize peptides on MHC class II molecules presented by antigen-presenting cells (APCs), and CD8+ T cells recognize peptides on MHC class I molecules which can be presented by all nucleated cells [2]. Naive CD4+ T cells respond to stimulation by differentiating into different T helper (Th) subsets, such as Th type 1, Th2, Th17, follicular helper T cells and regulatory T cells [8]. The strength of the TCR signal and the cytokine environment will determine which type of helper CD4+ T cell that is developed [9, 10]. The different lineages can be

distinguished by their transcription factors and production of cytokines. Th1 produces interferon (IFN) γ, interleukin (IL)-12 and tumor necrosis factor (TNF), and these cells are important boosters of macrophages and CD8+ T cells. Th2 effector cells can also produce TNF, but these cells mostly produce IL-4, IL-5 and IL-13, and the secretion of these cytokines are important for IgE antibody secretion [10]. Th17 are important in the response against extracellular bacteria and fungi and these effector cells produces IL-17, IL-21 and IL-22 [11]. Naive CD8+ T cells are specialized to become cytotoxic effector T cells that can directly kill infected cells that are infected with intracellular pathogens.

Activation of T cells by antigen bound to MHC molecules initiate a cascade of intracellular signaling events leading to T cell activation, including transcriptional activity and proliferation. The TCR α and β chains have short cytoplasmic tails, but the CD3 chains have long cytoplasmic tails that enables the TCR/CD3 complex to induce downstream signaling. The CD3γ, δ, ε and ζ-chains contain immunoreceptor tyrosine-based activation motifs (ITAMs) which are essential for TCR-mediated activation [12]. In response to TCR stimulation, the tyrosine residues within these ITAMs are phosphorylated by lymphocyte-specific tyrosine protein kinase [13]. The ITAMs recruit the Syk family kinase ζ-chain associated protein 70 kDa (ZAP-70). Lck and Zap-70 elicit full activation of downstream signaling by phosphorylating the adaptor proteins – linker for activation of T cells (LAT) and Src homolog domain-containing leucocyte-specific phosphoprotein of 76 kDa (SLP-76) [14, 15]. This induces the recruitment of various effector proteins involved in T cell activation and the formation of the LAT-SLP-76 signaling complex [16]. This complex is necessary for further downstream signaling events. Other second messengers such as diacylglyceros (DAG) and Ins(1,4,5)P3 and increased intercellular concentration of Ca^{2+}, will initiate activation of transcription factors leading to transcription of genes that direct T cell proliferation and differentiation [17, 18].

To get full activation of T cells, it is necessary with a second costimulatory signal. CD28 is the primary costimulatory receptor, but also other receptors, such as CD2 and inducible T cell costimulatory (ICOS), can give a costimulatory signal (ideally together with CD28) [16, 19, 20]. CD28 binds to the co-stimulatory molecule B7 (CD80 or CD86) on APC and CD2 interacts with LFA-3 (CD58) on APC. These signals are essential in order to facilitate a productive immune response. CD28 signaling is important for the IL-2 production, and T cell

activation without co-stimulation has been shown to induce anergy and promote T cell tolerance [21, 22].

Cytotoxic T-lymphocyte antigen 4 (CTLA-4) is another member of the CD28 family and is a negative regulator of T cell activation. CTLA-4 is very important for maintaining T cell homeostasis and is upregulated after activation. When it binds to B7, it will give an inhibitory signal to the T cell which will dampen the activation of the cell [18].

1.1.3 Acute and chronic inflammation

Inflammation is a protective response that can be triggered by a variety of harmful stimuli, including infections and injuries, and can be divided into acute or chronic and local or systemic. The initial response is called the acute inflammatory response which is a rapid non-specific response that triggers recruitment of immune cells [24, 25]. Inflammatory inducers can be exogenous signals such as pathogens and toxins but also endogenous signals as a result from tissue stress, injury and malfunction. Cells that sense these signals such as tissue-resident macrophages and mast cell will detect the inducers with specific receptors on their cell surface and will respond by producing inflammatory mediators. Different amounts and combinations of mediators will be produced depending on different inducers but usually this will result in restoration of the tissue homeostasis by promoting elimination of the inducer, and adaption of the noxious state [23]. An inflammatory response is characterized by the sensation of heat which is caused by increased movement of blood, and increased redness due to the increased number of red blood cells in the inflammatory tissue. Increased passage of fluid and infiltration of cells into the damaged area can cause swelling. Pain can also occurs due to the direct effects of mediators, either from initial damage or as a result of the inflammatory response [24].

In contrast to acute inflammation, chronic inflammation is a slower and long-term inflammation which can persist for weeks, months, years or even a life time, but the extent and effect varies depending on the cause of the injury and the body's ability to repair and overcome the damage. Chronic inflammation can occur after failure of eliminating a pathogen that is able to resist the host immune defenses and remain in the tissue for an extended period such as HIV infection, but also Hepatitis B and C and herpes viruses. It can also be a result of exposure to a particular irritant or foreign material which fails to be eliminated by enzymatic

breakdown or phagocytosis such as chemical substances that have been inhaled over a long period. Autoimmune diseases where the immune system attacks the body's own healthy tissue can give rise to chronic inflammation.

During chronic inflammation, most of the features of acute inflammation continue including expansion of blood vessels, increased blood flow and migration of neutrophils into the infected tissue. As the inflammation persist, macrophages and lymphocytes will replace the neutrophils and produce inflammatory cytokines, growth factors and enzymes. Eventually, the persistent inflammatory inducer or the responses by immune cells will cause tissue damage [25].

1.2 HIV-1

Human immunodeficiency virus (HIV) is a retrovirus that belongs to the lentivirus family. The virus was transferred to humans in the early 1900s [26]. For humans, two viral strains are pathogenic, namely HIV-1 and HIV-2. These strains share similarities regarding viral entry, replication and transmission, but they are different with regards to epidemiology, clinical outcome and molecular variations [27]. HIV-1 is mostly responsible for the worldwide pandemic, whereas HIV-2 is confined to areas of West-Africa and is considered less virulent [28, 29]. HIV-1 is the strain studied in this Thesis and will therefore be the focus in this introduction (hereafter simply termed HIV).

HIV became apparent in 1981 when previously healthy young homosexual men in the United States were diagnosed with *Pneumocystis pneumonia*, Kaposi sarcoma, mucosal candidiasis, cytomegalovirus infections and defects in cellular immunity [30, 31]. The same diseases and immune defects were also seen in intravenous drug users. The term acquired immune deficiency syndrome (AIDS) was used to describe these common conditions and it was quickly established that an infectious agent had to be the cause.

In 1983, HIV was isolated, cloned and genome sequenced, and it was concluded that HIV caused AIDS [32]. Development of the first highly effective therapy was licensed in 1987, followed by other medications with improved treatment response in the following years [33, 34].

Still today, more than 30 years since the first isolation of the virus, HIV continues to be a significant global health issue. Since the start of the epidemic, an estimate of 78 million persons have been infected and over 35 million are living with the disease [35]. The latest statistics estimated that approximately 20 million people worldwide were infected in 2017 and 1 million people died from AIDS-related illness in 2016 [36, 37].

1.2.1 The Virus

HIV, being a retrovirus, has RNA as the genetic material. The hallmark of retroviruses is the viruses' ability to reverse transcribe their RNA to complementary DNA (cDNA) and incorporate it into the cellular DNA of the host [38]. Considerable genetic diversity is seen in HIV variants and therefore four different groups are classified: M (main), O (outlier), N (non-M, non-O) and P (a newly analysed HIV sequence, called "pending the identification for further human cases"). Around 98 % of HIV infected persons worldwide are infected with HIV group M. The rare groups N, O and P have little or negligible global significance and are primarily restricted to Cameroon and surrounding countries [39]. Group M is further divided into subtypes A-K. Subtype B is the most prevalent subgroup in Europe and North-America, whereas group C is most seen in sub-Saharan Africa and India.

The structure of the HIV virus is similar to other retroviruses. It is surrounded by a lipoprotein membrane where the HIV envelope spikes are anchored. These envelope spikes comprise of three external glycoproteins (gp120) and three transmembrane proteins (gp41) subunits (Fig. 1). The two copies of single-stranded RNA are inside the nucleoprotein core (capsid) together with reverse transcriptase and integrase [40]. The virus only depends on three genes called gag (group-specific antigen), pol (polymerase) and env (envelope). The Gag and env genes code for the nucleocapsid and the glycoproteins of the viral membrane. Pol genes codes for reverse transcriptase and other enzymes. In addition, HIV contains six genes (vif, tat, rev, vpu, vpr and nef) which encode proteins necessary for host cell regulation and viral gene expression [41].

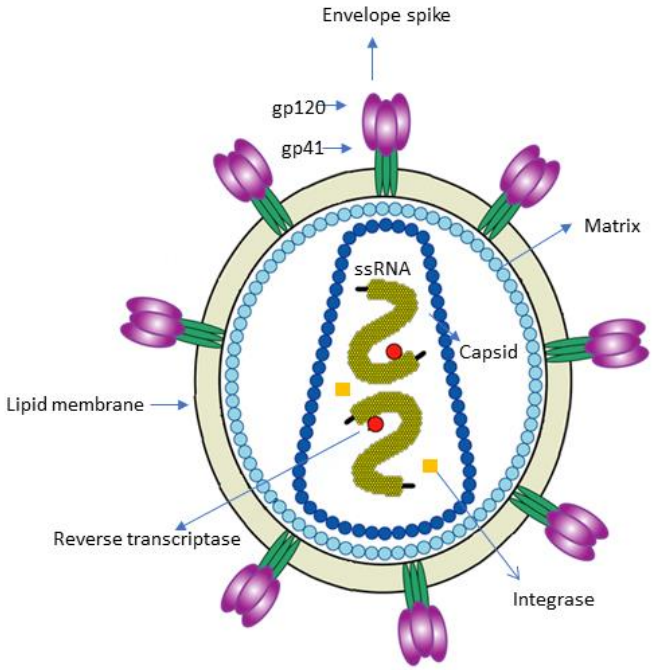


Figure 1. Structure of HIV virion. *Modified from www.niaid.nih.gov.*

The initial step of the HIV infection is binding to the target cell. HIV can either infect cells by releasing cell-free virions through the extracellular fluid or by direct cell-to-cell routes between infected and uninfected cells [42]. When bound to a cell, the gp120 of the envelope spike binds to the CD4 receptor on the target cell. The attachment leads to a conformational change in the CD4 molecule which opens up the binding site for gp120 to the co-receptors C-C chemokine receptor type 5 (CCR5) or C-X-C chemokine receptor type 4 (CXCR4). Binding of both CD4 and the co-receptor on the cell surface leads to an additional conformation change in gp41, which results in membrane fusion and delivery of the viral content into the host cell [43]. In the cytoplasm, reverse transcriptase begins the synthesis of double-stranded viral DNA. The capsid will now gradually dissolve and the viral DNA together with viral and host proteins forms a pre-integration complex [44]. PIC is transported into the cell nucleus via nucleoporins. In the nucleus, viral integrase cuts the host chromosome and inserts viral DNA at random and establishes the so called provirus [45]. The host cells' activation status decides if the virus establishes a productive infection or a latent infection. Rapid and efficient replication occurs in activated T cells, but in quiescent T cells, the provirus remains in an inactive state until the host cell starts to proliferate [46]. When the provirus has been transcribed, two copies of viral RNA genome are packaged into a nascent virion. The structural components such as the matrix, capsid and enzymes are established by

cleaving of the Gag and Gag-Pol precursor proteins by the HIV protease. The viral particle is now ready to leave the host cell through the host cell endosomal sorting complexes required for transport into the extracellular space, ready to infect a new cell [47, 48].

HIV is dependent on the CD4 receptor to enter cells, so the main targets of HIV are CD4+ T cells which also express CCR5 and CXCR4. Other cells that can express the CD4 receptor and the co-receptors are monocytes, macrophages and dendritic cells but these cells are not always expressing CD4 and are not as frequently infected as CD4+ T cells [43]. DCs express relatively low levels of the CD4 receptor, but the ones who do normally get infected in the vaginal and rectal submucosa. These areas are rich in DCs and HIV infection commonly occurs through the vaginal or rectal routes. Infected DCs can contribute to the spread of the infection by interacting with CD4+ T cells via infectious synapses [49].

The acquisition of the infection is highly dependent on the co-receptor CCR5. Persons who are carriers of the CCR5 delta32 mutation (a mutation that results in lack of expression of the receptor) are highly resistant to HIV-1 infection, as the virus cannot enter the host cell without a functional co-receptor [50]. Homozygous carriers of this mutation are primarily found in the white population in Northern Europe. In Scandinavia, 2 % of the population is homozygous for this mutation. Persons that are heterozygous for the mutant have a reduction in functional CCR5 receptors and show a slower disease progression and have prolonged survival, if infected with HIV-1 [51].

1.2.2 Course of untreated disease

Immediately after being infected with HIV the virus starts to replicate. After 10-14 days the acute phase of the infection begins when the majority of patients experience a “flu-like” febrile illness such as fever, malaise, sore throat, headaches, nausea etc [52]. In this phase the plasma viremia increases rapidly and reaches extreme values and the number of circulating CD4+ T cells drastically declines (Fig. 2) [52, 53]. Typically the symptoms subside within two weeks when virologic control has been established, but this may vary from a few days to several months. Now the patient enters the asymptomatic phase (latency phase) of the disease which can last several years but this depends on the patient’s clinical outcome [54]. The immune responses against the infection will decrease the viral loads which will reach a stable level known as the viral set point, and the CD4+ T cell number increases [55].

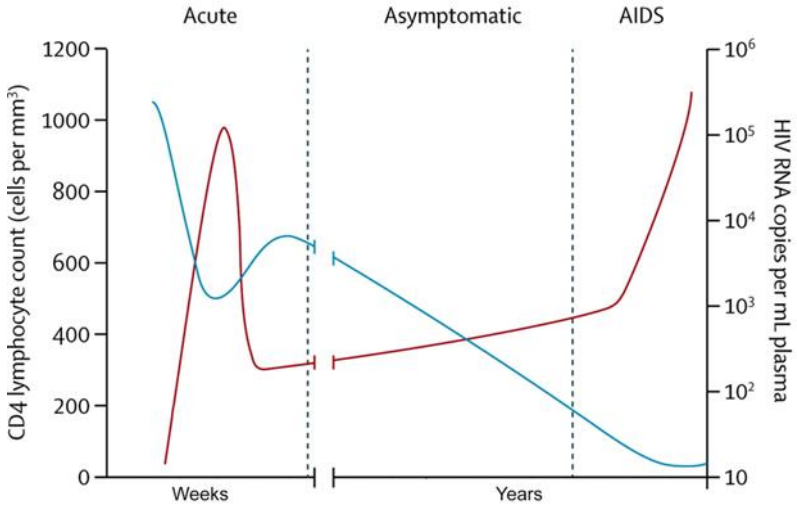


Figure 2. Course of an untreated HIV infection. *In the first weeks of infection the viral load drastically increases (red line) resulting in a rapid and significant loss of CD4+ T cells (blue line). After a few weeks the asymptomatic phase begins where the immune system regain some control of the infection and viral load decreases and CD4+ T cells recovery to some extent. In this phase most patients have modest symptoms. As the disease progresses, immune control is gradually lost and CD4+ T cells steadily declines. Eventually the patient reaches the end-stage of HIV, known as AIDS. Modified from [43].*

Even though some control is establish in the latency phase, viral replication continues resulting rising viremia and in gradually loss of CD4+ T cells. Eventually, the viral control is lost due to the critically low number of CD4+ cells and the patients enters the end-stage where AIDS is developed [35].

Without treatment, the disease will usually progress to AIDS, but the rate of progression can vary greatly. The disease progression can be divided into three groups depending on CD4+ T cells counts before treatment: rapid progression, intermediate progression, and long-term non-progression. Patients with a rapid progression will develop AIDS within three years after being infected with HIV [56]. Intermediate progression, which is the most common, will result in development of AIDS after 3-10 years [54]. A minority of patients maintain high CD4+ T cell counts (above 500 cell/mm3) for many years after infection. These are referred to as long-term nonprogressors. These patients are free of symptoms and do not need treatment for more than ten years after being infected with HIV [57]. Patients in the long term non-progression group who also show RNA viral loads below the detection limit are called “elite controllers” [58]. These patients are of high interest in the research communities in order to understand how they control the virus and to take advantage of those properties in a treatment setting.

1.2.3 AIDS

For almost all untreated HIV+ patients, a progression of the disease occurs and eventually leads to AIDS. AIDS is characterized by immune deficiency that not only affects HIV-specific immune responses but also responses to other pathogens and malignancies. AIDS is developed when the immune system is too weak to fight off opportunistic infections or diseases such as cancer. The median time from an HIV+ patient develops AIDS to the patient dies from opportunistic diseases is two years [38].

1.2.4 Antiretroviral therapy

The major approach to treat HIV infection is with ART. Current ART regimens suppress plasma HIV viraemia to below limit of detection, increases the number of CD4+ T cells, improves memory T cell responses and decreases immune activation [59]. ART has turned HIV into a chronic disease which is kept stabile and manageable when treated and have radically improved the life expectancy of patients infected with HIV and reduced HIV morbidity and mortality [36]. The treatment has also reduced transmission of HIV since it reduces the viral load in HIV+ patients [60]. By combining drugs that attacks the virus in different ways, the therapy prevents the virus from reproducing itself and limits the virus' possibilities to develop resistance by inhibiting several critical steps in the viral life cycle [63]. There exist six classes of anti-HIV therapies with several drugs in each class:

Table 1. The six classes of anti-HIV therapies available.

Drug classes	Basic principles of the drugs
Nucleoside reverse transcriptase inhibitors	Blocks reverse transcriptase and thereby inhibits the production of cDNA [61].
Non-nucleoside reverse transcriptase inhibitors	Inhibiting reverse transcriptase by binding to an allosteric site on the enzyme [62].
Protease inhibitors	Inhibits cleaving of Gag and Gag-Pol precursor proteins by blocking HIV protease [64].
Integrase strand transfer inhibitors	Prevents the HIV genome from being integrated into the host genome [63].
Entry inhibitors	Binds CCR5 preventing gp120 to bind this co-receptor (these drugs require some technical testing to ensure that the patient do not have virus variants that uses the co-receptor CXCR4) [63].
Fusion inhibitors	Binds to the gp41, preventing fusion of the viral membrane with the host membrane [63].

The initial anti-HIV therapy drugs were established in the mid-1980s when nucleoside reverse transcriptase inhibitors (NRTIs) were developed. The first single-drug therapy (monotherapy) was the drug Azidothymidine, originally developed for anticancer treatment [64]. Shortly after, non-nucleoside reverse transcriptase inhibitors (NNRTIs) were developed, but these monotherapies were vulnerable to mutations in the HIV genome leading to early drug resistance [65]. When protease inhibitors (PIs) were approval in the mid-1990s, treatment was dramatically improved by combining drugs that target both reverse transcriptase and protease to enhance the overall efficiency and durability of the therapy [66]. ART revolutionized the HIV treatment by introducing dual- and triple drug combinations to prevent drug-resistant. Early clinical trials of ART showed that three drug combinations from at least two different

drug classes decreased probability of selecting resistant viral clones. The current classes of drugs included in ART are listed in Table 1. The regimens of drug combinations of NRTIs, NNRTIs and/or PIs are most common. Integrase, entry, and fusion inhibitors are newer classes of drugs and are also used in combination with NRTIs, NNRTIs and PI, especially to treat individuals harbouring multidrug-resistant virus [67].

Current treatment cannot suppress viral replication and cell-to-cell infections entirely because of latent HIV reservoirs resulting in a rebound of HIV replication if treatment is stopped. Integrated HIV provirus, which is transcriptionally silent, is more or less impossible to detect, and is stable for years due to the long life of many of the latency infected immune cells [68]. The HIV provirus reservoir has been shown to establish within the first days of infection, even before the increased plasma viremia can be detected. Mostly resting CD4+ T cells are infected but also monocytes, macrophages and DCs can be used as reservoirs [46, 69]. Resting CD4+ T cells can either be naive or memory T cells and both of these populations are susceptible to get infected. Memory CD4+ T cells can live for a very long time and are ideal reservoirs for the virus. In contrast to activated CD4+ T cells, only a fraction of memory CD4+ T cells express CCR5, and most of the naive T cells do not express it at all. Demonstrated by the resistance to HIV infection seen in individuals which are homozygous for the CCR5 delta32 mutation, studies have shown that during early transmission of the infection, the virus is dependent upon CCR5 co-receptor binding. HIV usage of the co-receptor CXCR4 usually emerge later in the course of the disease. This indicates that infection of many memory CD4+ T cells occur only in a small subset of memory T cells that do express CCR5, but it has also been observed that when the cells are in the process of reentering the resting state after activation they can be infected and carry with them a copy of the HIV genome. The infection in resting CD4+ T cells are nonproductive until the cell is reactivated by a cognate antigen. Since naive T cells do not express CCR5 they might be infected through mechanisms that do not involve direct infection. One possibility is that some resting memory cells with integrated HIV-DNA might revert to the naive phenotype. Another possibility is infection of thymocytes resulting in infected T cells before they become mature naive T cells [70].

Monocytes and macrophages can also be used as a latent HIV reservoir, but this is seen in a much less extent compared to reservoirs in CD4+ T cells. This is due to the generally lower

expression of CD4 on monocytes and macrophages, but infected monocytes and macrophages can also contribute the HIV reservoirs by spreading the virus to CD4+ T cells [71].

Even though ART has drastically improved the life expectancy of HIV+ persons, many clinical challenges remain. HIV+ patients treated with ART have an elevated risk of typically age-related non-AIDS conditions, such as cardiovascular disease, cancer, osteoporosis, diabetes, kidney diseases, liver diseases, and bone fracture [72-76]. Serval factors are related to this increased risk of non-AIDS diseases, such as long-term toxic effect of ART drugs, and the chronic inflammation seen in HIV+ patients. Also a higher prevalence of traditional risk factors such as tobacco, alcohol and other substance abuse is also often linked with the increased risk [77].

1.2.5 Immunological nonresponders

A proportion of patients do not restore their CD4+ T cell counts to normal levels despite long-term ART and sustained viral suppression. Compared to patients who do have sufficient immune restoration, these co-called immunological nonresponders (INRs) have an even higher risk of AIDS-related and non-AIDS related morbidity and mortality [78-80]. The prevalence number of INR patients varies from 15-30 % because the definition of INR suffers from lack of consensus. Some studies define INR patients with CD4+ T cell count < 400 cell/μL, other are more strict and defines INR patients with CD4+ T cell count < 200 cells/μL [81]. The basis for INR development is not completely understood, but some studies associate it with low nadir CD4+ T cell count, older age when infected, altered thymic production, and co-infections such as hepatitis C [81, 82].

1.3 Chronic inflammation during HIV pathogenesis

Chronic inflammation and immune activation is highly associated with the pathogenesis of untreated HIV infection. During acute infection, rapid and high production of cytokines such as IFNα, IFNγ, inducible protein 10, TNF, IL-6 and IL-10 are seen, together with a dramatic increase of activated CD8+ T cells. ART will reduce the viral replication to negligible levels, but ART cannot eradicate the virus leading to a low but persistent chronic viral replication. The cause of this inflammation seen in both untreated and treated HIV+ patients is probably multifactorial. Mucosal damage leading to microbial translocation is incompletely reversed in

patients on ART, and is a central factor driving inflammation [83]. Co-infections, such as with cytomegalovirus, latent herpes virus and varicella zoster virus are commonly seen in HIV patients, and are associated with increased levels of T cell activation. Also, chronic inflammation has been associated with dysfunction of the thymus and regenerative failure of CD4+ T cells [84].

LPS in the circulation system will cause inflammation and trigger production of pro-inflammatory cytokines such as IL-6. IL-6 is released from monocytes and macrophages, and HIV infected patients usually have increased levels of IL-6 in plasma compared to uninfected controls. IL-6 is strongly associated with all-cause mortality [85].

The collective outcome of these pathways is a persistent inflammatory state. The pro-inflammatory cytokine IL-6 and the protein D-dimer is seen in increased levels for both untreated and treated patients [86]. LPS will bind to CD14 receptor expressed on monocytes and macrophages which results in cleavage of the CD14 into soluble CD14 (sCD14) which is released into the circulation. sCD14 can bind circulating LPS which will activate several types of immune cells [87]. CD163 is another receptor expressed on the surface of monocytes and macrophages which in response to toll-like receptor activation will be cleaved off and released as sCD163 and bind LPS [88]. sCD14 and sCD163 are elevated in HIV and can be used as a measurement of immune activation and inflammation.

1.3.1 HIV in the gut leads to systemic inflammation

The gut is an antigen-rich environment that contains high numbers of activated memory CD4+ T cells compared to the peripheral blood and lymph nodes [89]. CCR5 which is a co-receptor used by HIV, is highly expressed on these CD4+ T cells because of consistent activation. HIV replicates more efficiently in activated memory CD4+ T cells, and during early HIV infection high numbers of CD4+ T cells in the gut-associated lymphoid tissue (GALT) is depleted. The depletion of CD4+ T cells occurs by direct killing by cytotoxic T cells, but high numbers of uninfected CD4+ T cells are also victims of bystander killing directly by CD8s or indirectly by apoptosis and pyroptosis. Pyroptosis is a form a programmed cell death which is a major cause of CD4+ T cell depletion. Cytosolic accumulation of incomplete HIV reverse transcripts and proinflammatory cytokines such as IL-1β both trigger an immune response which leads to caspase-1 activation and pyroptosis [90, 91]. The gut mucosal surfaces contain high levels of Th17 T cells secreting IL-17 and IL-

22, which are important in the defense against microbes and for maintaining gut epithelial integrity [92]. Depletion of these cells results in leakage of microbial products from the intestinal lumen into the circulation (Fig. 3), causing systemic immune activation [83].

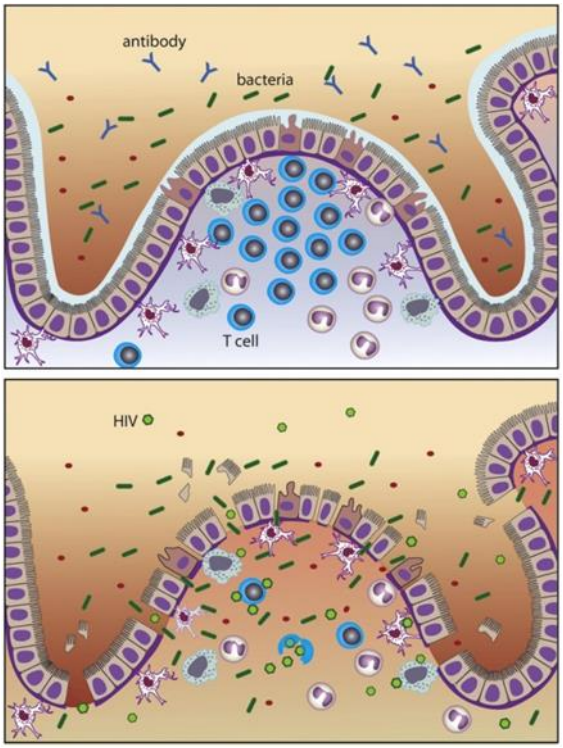


Figure 3. Impact of HIV on gut mucosa. *A healthy gut mucosa protects the surrounding environment from microbes and microbial products (upper image). When HIV infection occurs, the depletion of CD4+ T cells in the gut mucosa leads to breakdown of the tight junctions in the mucosa, microbial translocation and further dysregulation of resident immune cells (lower image) [77].*

Leakage of microbial products such as LPS triggers a systemic immune response by activating monocytes and DCs and induces secretion of pro-inflammatory cytokines such as IL-6, TNF and type 1 IFN [93]. Elevated levels of LPS are associated with T cell activation and poor CD4+ T cell recovery. ART might dampen this process but incomplete effects are seen in many treated HIV patients [94].

Microbial translocation is also seen in other diseases such as inflammatory bowel disease, pancreatitis, obesity and diabetes [95-97]. This translocation of microbial products can in all these disease cause local inflammation. After passing the liver microbial products can cause systemic inflammation via their stimulation of innate immune cells [98]. Unlike the other diseases, HIV infection damages the gut mucosa in two different ways. In addition to the massive depletion of CD4+ T cells in the gut mucosa, the enzyme called indoleamine 2,3-dioxygenase (IDO) also contributes to HIV-related immune dysfunction by inhibiting Th17

cell differentiation. IDO, which is an interferon- and microbial product-inducible enzyme, catabolizes the amino acid tryptophan in innate immune cells such as macrophages and DCs. Inflammation and IFNγ often correlate with increased IDO expression but LPS and TNF can also enhance the expression without the presence of IFNγ [99]. Systemic IDO activity will decrease the level of tryptophan and increase the levels of tryptophan catabolites such as picolinic acid and kynurenine. This process can lead to inhibition of effector T cell proliferation capacity [100]. Also activation of the same system leads to increased FoxP3 expression which will suppress the secretion of IL-17 in CD4+ T cells resulting in a shift from Th17 cells into Tregs [101]. Despite ART, this process is not normalized in HIV patients. Th17 cells which secrets IL-17 enhances the host defense against microbial agents and loss of these cells are thought to comprise the gut barrier which will increase the microbial translocation and systemic inflammation [101].

1.3.2 Activation and differentiation of T cells

Chronic inflammation is a hallmark of HIV infection and is observed by increased activation of T and B cells, increased levels of circulating inflammation markers and is a driver of disease progression [102]. ART decreases this chronic activation but long-term ART treated patients still show a low-grade inflammation and immune activation [103].

Immune activation can be defined by CD38 and HLA-DR expression on T cells, and this is a widely used marker for HIV progression and mortality. CD38 is a transmembrane glycoprotein which can be expressed in both hematopoietic and non-hematopoietic cells but its expression varies during lymphocyte development, activation and differentiation. On T cell precursors and double positive CD4/CD8 T cells CD38 is highly expressed. Mature naive T cells express low levels of CD38, but the expression is upregulated upon antigen stimulation [104]. CD38 is a transmembrane protein that is important in lymphocyte activation. It functions as an enzyme for the synthesis of CD^{2+} [105, 106]. HLA-DR which is a MHC class II molecule, can also be expressed on T cells upon activation. In HIV infection the expression of HLA-DR increased on CD8+ T cells upon activation and stays relatively stable throughout the disease progression, whereas CD38 expression continues to increase throughout the disease progression and function as a marker of future CD4 loss. Expression of HLA-DR on T cells can also be seen in several autoimmune diseases and aging [107].

CD45 is a glycoprotein that is expressed by lymphocytes. Multiple isoforms of this molecule exist and are differentially expressed depending on the cell type and activation status [108]. By alternative splicing of several exons, the different CD45isoforms are generated. Naive T cells express the high molecular weight isoform containing the A exon (CD45RA) but this isoform is lost when the T cell is activated and is replaced by the lower molecular weight isoform CD45RO. Upon an infection T cells have an important role in generating effector cells that mediate antigen control and by forming memory T cells that are longed-lived and will provide protective immunity against recurring infections. In peripheral tissue, memory T cells confer rapid immune responses to secondary antigenic stimulation. Effector memory T cells (T_{EM}) provide protective immediate effector functions in inflamed areas, while central memory T cells (T_{CM}) localized in the lymphoid tissue have little effector functions, but are ready to react to a secondary infection [109]. Another subgroup of memory T cells is called terminally differentiated effector memory cells (TemRA) which are different from EM and CM T cells by their re-expression of CD45RA. TemRA cells have been shown to have less proliferation capacity and cytokine production. They have mostly been studied in the CD8+ T cell population, but they are also found among CD4+ T cells. However, in contrast to TemRA CD8+ T cells, the frequencies of TemRA CD4+ T cells vary drastically between individuals [110]. Persistent viral infections, inflammatory conditions and ageing all induce the accumulation of highly differentiated CD45RA re-expressing memory T cells. The cell surface receptor CD27 is a member of the TNF receptor superfamily. CD27 binds to its ligand CD70 and has been shown to enhance T cell proliferation indicating that it has a role in regulating T cell activation [111]. Together with CD45RA it can be used phenotypically to define subsets of naive and memory T cells. Naive and CM T cells expresses CD27, while EM and TemRA T cells do not [112].

In HIV infection, HIV-specific CD8+ T cells, also called cytotoxic T lymphocytes (CTLs), respond to HIV epitopes, such as Gag. In the beginning of the HIV infection, CTL responses are associated with lower viral loads but CTL functions are often lost during the chronic phase the infection. This is due to T cell exhaustion and reduced help from CD4+ T cells [113, 114]. HIV elite controllers have been shown to have increased HIV-specific cytotoxic T lymphocyte responses to Gag epitopes and broader repertoire of effector mechanisms [115]. In response to gag epitopes displayed on MHC class I molecules, CD8+ T cells will produce several cytokines such as IFNγ, TNF and IL-2. IFNγ is the only member of the type II class of interferons, and is very important in the CD8+ T cell responses against infections. Production

of IFNγ will increase the stability and expression of MHC class I molecules and it can also induce apoptosis by the Fas/Fas ligand pathway. TNF is a member of the TNF superfamily and is also shown to be important in antigen responses by mediating direct apoptosis through the TNF type 1 receptor as well as promoting an antiviral state such as through IFNγ by enhancing expression of MHC class I molecules. IL-2 has no direct antiviral effector functions but it is an important primary growth factor for T cells and central for inducing rapid expansion of effector cells in an ongoing immune response [116]. Cytokines can also be important in immune regulation. TGFβ which is a multifunctional cytokine is a member of the transforming growth factor superfamily. Production of TGFβ promotes the survival of peripheral T cells, but it also inhibits T cell proliferation and differentiation, and has been associated with HIV progression [117, 118]. These same cytokines are also produced by CD4+ T cells. In HIV infection, CD4+ T cells contribute to the activation of HIV-specific CTLs but the CD4+ T cells` capacity to proliferate and produce cytokines are reduced since they are the main target of the virus [119].

1.3.3 Exhaustion: PD1, Lag3, Tim3, TIGIT and CD160

Upregulation of co-inhibitory molecules on T cells will result in negative feedback mechanisms. By suppressing signaling pathways in T cells, these co-inhibitory molecules may induce reversible or irreversible T cell inhibition, dysfunction and apoptosis [120]. Co-inhibitory molecules are vital for maintaining self-tolerance and to down-regulate immune responses when a pathogen is cleared in order to minimize tissue damage. In chronic diseases such as HIV and cancers the upregulation of co-inhibitory molecules on T cells is seen more frequently and can result in the effector T cells becoming exhausted [121]. Exhaustion is associated with reduced cytokine production, killing capacity and proliferative potential.

Upon activation, together with positive regulators of T cell activation, negative regulators will be upregulated. CTLA4 is one of the first negative regulators to be induced and directly competes with the co-stimulatory molecule CD28 for the ligands CD80 and CD86 on APCs [122]. There are several other molecules that give an inhibitory signal to the T cell, such as programmed cell death protein 1 (PD-1), lymphocyte activation gene-3 (Lag-3), T cell immunoglobulin-3 (Tim-3), T cell immunoreceptor with Ig and ITIM domains (TIGIT), and CD160.

PD-1 is also a member of the CD28 family but in contrast to CD28 and CTLA-4 which share the same ligands, PD-1 interacts with the ligands PD-L1 and PD-L2. PD-1 can be expressed on CD4+ and CD8+ T cells but also on B cells, and most APCs. Resting T cells do not express PD-1, but it is induced when T cells are activated. PD-L1 and PD-L2 are mostly expressed on T and B cells, macrophages, DCs but PD-L1 can also be expressed on a variety of non-hematopoietic cells [123]. The cytoplasmic region of PD-1 has two tyrosine residues. One of these is membrane-proximal which constitutes an immunoreceptor tyrosine-based motif (ITIM) whereas the other is a part of the immunoreceptor tyrosine-based switch motif (ITSM). The ITSM is important for the inhibitory function PD-1 has [124]. The two tyrosine residues are phosphorylated upon ligation with PD-L1 or PD-L2 which lead to binding of SH2-domain containing tyrosine phosphatase-1 (SHP-1) and SHP-2. This leads to downregulation of the TCR through dephosphorization of signaling intermediates [125, 126].

LAG-3 is another co-inhibitory molecule that can be upregulated on activated effector CD4+, CD8+ T cells, and NK cells [127]. The structure of Lag-3 resembles the CD4 co-receptor and Lag-3 can bind MHC class II with a higher affinity than CD4. Lag-3 inhibits T cell proliferation, cytokine production and calcium flux as crosslinking of Lag-3 together with CD3 inhibits downstream signaling. How Lag-3 crosslinking to CD3 inhibits downstream signaling remains elusive [128, 129].

TIM-3, another negative regulator of immune responses, is mostly expressed on IFN-γ producing CD4+ T helper 1 and CD8+ T cytotoxic T cells, but has also been identified on Tregs and DCs, NK cells and monocytes. In healthy persons, expression of Tim-3 seems to regulate Th1 responses, and the induction of peripheral tolerance [130]. Tim-3 does not have classical signaling motifs on its cytoplasmic tail but instead has 5 tyrosine residues. When Tim-3 interacts with its ligands galectin-9 or Ceacam-1, these tyrosine residues will be phosphorylated by Src family kinases leading to downstream signaling inhibition. Elevated expression of Tim-3 is observed on T cells in HIV infected persons which will lead to dampening immune responses with decreased proliferation capacity and cytokine production [128, 131].

TIGIT is another co-inhibitory receptor which belongs to the immunoglobulin (Ig) superfamily [132]. TIGITs two ligands, CD155 (PVR) and CD112 (PVRL2, nectin-2), are expressed on dendritic cells, and non-hematopoietic cells [128]. TIGIT has an extracellular IgV domain, a transmembrane domain and a cytoplasmic tail containing two ITIMs and

competes with CD226 (DNAM-1) and CD96 (Tactile) for the ligand. The pathway is very similar to the CD28/CTLA pathway, where CD226 will induce a positive signal (such as CD28) and TIGIT and CD96 will induce a negative signal (such as CTLA-4) [132]. Signaling through TIGIT and PVR will recruit phosphatases via TIGITs intracellular ITIM domain and lead to suppressed TCR signaling [133].

CD160 is another negative co-stimulatory receptor which is a member of the B7/CD28 family. It is expressed on cytotoxic cells such as NK cells, NKT cells, CD8+ T cells and on a small subset of CD4+ T cells. When CD160 binds to its ligand HVEM it will inhibit T cell proliferation and cytokine production. CD160 is also able to bind to MHC class I molecules with a low affinity. HVEM can act as both a ligand and a receptor and has several binding partners such as BTLA which is another negative co-stimulatory receptor. By binding to different receptors such as CD160 and BTLA, inhibitory signals are produced. If HVEM binds to LIGHT/TNFSF14, positive signals are produced that activates T cells [134, 135].

1.3.4 Senescence: CD57 and KLRG1

Immunosenescence or loss of immune cell function occurs when T cells and other leukocytes eventually no longer can respond to pathogens. Senescence of T cells is common during aging for all individuals, but HIV+ patients usually experience senescence much earlier than persons not infected with HIV. It is the constant triggering of the immune system, and the chronic inflammation in HIV+ patients that accelerate aging in HIV disease [136].

Senescent T cells will downregulate their co-stimulatory molecule CD28 and upregulate CD57, and the cell telomere lengths will be shortened. Their capacity of producing IL-2 will also decrease which result in lowered proliferative capacity. CD28 is an important co-stimulatory molecule for the signaling cascade needed for T cell activation. When T cells downregulate this molecule, the T cells will lose not only their proliferation capacity but also their ability to help B cell proliferation [136].

Killer-cell lectin receptor G1 (KLRG1) is expressed on antigen-experienced T cells and NK cells and is also a marker associated with senescence [137]. Not only will the expression of KLRG1 dramatically increase with time, the expression also correlates with differentiation and is seen with the highest expression on memory T cells and other highly differentiated end stage cells. In acute HIV infection and other viral infection, KLRG1 can often be used to

distinguish between short-lived CD8+ T cells, which will have high expression of KLRG1 and memory effector CD8+ T cells which will have lower expression of KLRG1 [138].

1.3.5 Immune checkpoints inhibitors

Blocking the interaction between exhaustion molecules and their ligands can reverse T cell exhaustion and restore effective T cell responses. Several studies have demonstrated that blocking the interaction with specific Abs that targets for example PD1, PD-L1, Lag-3, and TIGIT have shown promising results for both viral infection diseases and cancers [139-142].

Anti-PD-1 has been used in treatment for many cancer patients to block the interaction between PD-1 and PD-L1, but patients developing resistance to therapy have been increasingly observed. One reason for this is that blocking PD-1 has been shown to result in upregulation of other immune checkpoints molecules such as Tim-3, Lag-3 and TIGIT and CD160. Several studies are now looking into the possibilities of targeting several immune inhibitory molecules at the same time to prevent resistance [143]. Dual blockage of PD-1 and TIGIT has been demonstrated in mouse model of chronic lymphocytic choriomeningitis virus infection and has shown to have a promising effect. It is suggested that this combination can be used to establish increased control in human viral diseases as well [139, 143, 144].

1.3.6 Gut homing of T cells: ITGβ7

GALT is a major homing site for CD4+ T cells, especially Th17 T cells. It is still not fully understood why so many CD4+ T cells are infected and killed by HIV in the gut, and why it is so poor reconstitution of these cells. However, as GALT is a tissue especially affected in HIV pathogenesis, gut-homing T cells are of great interest to study. To enter the gut mucosa, CD4+ and CD8+ T cells must express the integrin (ITG) α4β7. ITGs are heterodimeric α/β transmembrane cell adhesion receptors. They are connected to several biological responses such as cell migration, inflammatory response, tissue organization and target recognition by lymphocytes. The ligand to the gut homing receptor α4β7 is mucosal vascular addressin cell adhesion molecule 1 (MADCAM1), which is a cell surface adhesion molecule located in the vascular endothelium of the gastrointestinal tract [145]. HIV-1 envelope (gp120) is able to bind to ITG-α4β7 by imitating epitopes that MADCAM1 has. By binding to the ITG-α4β7 receptor, HIV-1 can enter the gut which is the primary site of virus replication [146].

1.4 Flow cytometry

Flow cytometry is a laser-based technology that is widely used for analyzing cell characteristics. The technology makes it possible to study multiple parameters of single cells, such as measuring the expression of cell surface markers and intracellular molecules. In this way, flow cytometry enables the analysis and categorization of different cell types in a heterogeneous cell population [147].

A single cell suspension is injected through the fluidics system together with sheath fluid (isotonic sheath fluid) and pushed through the instrument (Fig. 4). The sheath fluid is under pressure and will move slightly faster than the cell sample. This will lead to a drag effect where the stream of cells is surrounded by the sheath fluid that will make the central core narrower. This process is called hydrodynamic focusing and will result in a single stream of particles through an interrogation point, where one or more beams of light, usually coming from a laser, will cross the cells [148].

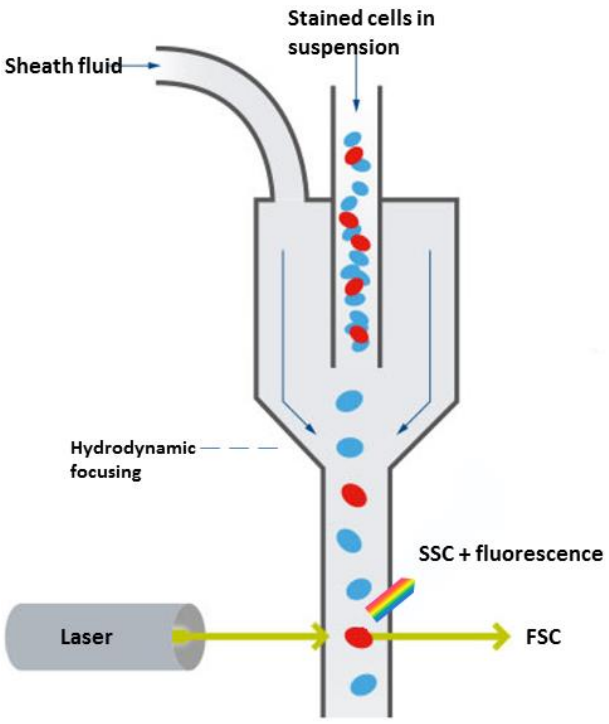


Figure 4 Principles of flow cytometry. *Fluorochrome-stained cells pass one at the time by one or more lasers in the flow cell. Modified from [149].*

The beams will scatter light and based of the cells scattering abilities, the light can provide information about each cell. Forward scatter measurements can distinguish cells by their size and side scatter can provide information of the cells' cytoplasmic granularity and internal structure. If the cells are stained with fluorochrome-conjugated Abs, the fluorescence from the cells will emit light when excited by the laser with the corresponding excitation wavelength. Different lasers produce a single wavelength of light, but lasers producing light of different wavelengths ranging from ultraviolet to red are available. To control the specificity of the detection, optical filters are used to block certain wavelengths while letting others pass through. Three filter types exist; long pass filter, short pass filter and band bass filter. All these filters block light by absorption. Mirrors are often used in the optical system in order to direct the light path [148, 150].

The scattered light and fluorescence signal from the cells will eventually encounter photomultiplier tubes (PMTs) that can convert the light signal into electronic pulses. A voltage supply will control the sensitivity of the PMTs. The extent of the electrons forming the electronic pulse is proportional to the number of photons that hit the photocathode and proportional to the light intensity that is scattered or fluorescence generated by the cell/particles. The electronic pulses coming from the very beginning when the cell or particles enter the laser beam to the cell/particles leave the beam, are called an event. However, not necessarily all of these pulses produced when a cell/particle passes the beams can be considered events, and it is therefore important to set a desirable "threshold" to cut out pulses that are produces by particles that are not considered as events such as signals from sources that are irrelevant to the experimental data. These sources can come from for example dust, debris, very small particles and light, and by deciding a threshold any pulses that fail to exceed the threshold level will be ignored. When the pulses from the cells are generated, the signals can be displayed as plots on computer programs where they can be analysed and interpreted [147, 148, 150].

2 Aims of the study

This Thesis is based on previous work performed in the Taskén group together with collaborators at Oslo University Hospital, where it was shown that HIV+ INRs patients have more activated and differentiated T cell phenotypes, compared to HIV+ patients with normal recovery of the CD4+ T cell pool (Stiksrud, Lorvik et al, 2016).

In this study, further analyses were executed on a new patient cohort which included INRs patients, patients who respond satisfactory to ART and uninfected persons. The aims of the Thesis were:

1. Analyse cell surface markers in order to investigate if there are differences in INRs patients compared to IR patients and healthy controls in regards to activation and differentiation.

2. Assess whether the INRs patients display a more exhausted and senescent T cell phenotype?

3. Establish a method to try to reverse T cell exhaustion (*in vitro*) in order to increase the CD4+ T cell levels and effector functions to INR patients.

3 Materials and Methods

3.1 Sample material

3.1.1 Patient material from study groups

Frozen peripheral blood mononuclear cells (PBMCs) from HIV+ patients were obtained from the Department of Infectious Diseases, Oslo University Hospital, Ullevål. Altogether, 52 samples were collected by study nurses. 19 samples were from HIV+ immunological nonresponders (INR), 17 from HIV+ immunological responders (IR) and 16 from healthy uninfected controls (HC). The cohort of participants who gave blood to this research were male patients in the age between 25-65 who had been on continuous ART for more than 4 years, and with HIV RNA under 50 copies/µL for at least 3,5 years (Table 5). Those participants with CD4+ T cell count < 400 cell/µL were defined as INRs, whereas patients with CD4+ T cells count < 600 cell/mL were classified as IRs. CD4 and CD8 T cell counts were provided from the Department of Immunology at Oslo University Hospital, Rikshospitalet. All participants had given their written informed consent, and the study was approved by the Regional Ethics committee. PBMCs were isolated, frozen and stored at -150°C at the Department of Infectious Diseases. At a later time the PBMCs were transported to Centre for Molecular Medicine Norway (NCMM), Oslo Science Park, where they were kept in a -150°C freezer until analysis.

3.1.2 Isolation of PBMCs from healthy blood donors

PBMC, are peripheral blood cells that have a single round nucleus such as lymphocytes (T and B cells and NK cells), monocytes and dendritic cells. These mononuclear cells were purified from buffy coats from healthy blood donors obtained from the Oslo University Hospital Blood Centre, Oslo, Norway. The buffy coat is a concentrated leukocyte suspension; it contains most of the white blood cells and platelets from a whole blood sample. LymphoprepTM (#12KAS03, Axis Shield), a density gradient medium, was used to isolate the mononuclear cells from buffy coats. The polysaccharides in the Lymphoprep will enhance erythrocyte and granulocyte aggregation which will increase their sedimentation rate through the Lymphoprep layer during centrifugation. Mononuclear cells have a low density and remain untouched and highly purified and will centre in a thin layer between the plasma and

the density gradient medium, where they were harvested with a pipette. The cells were then washed with PBS with 2 % fetal calf serum (FCS) and centrifuged at 300g, 4°C for 20 minutes. Samples that still contained red blood cells were lysed. The red blood cells were removed by using RBC Lysis Buffer 10X (#420301, BioLegend). Before use, the buffer was diluted 1:10 with MQ-H2O. The buffer will lyse the red blood cells because it contains ammonium chloride. For shorter time points, the lysis will have minimal effect on leukocytes. The lysis buffer was incubated with the cells for 10 min in the dark before washing with PBS with 2 % FCS and centrifugation.

The cell pellets were then resuspended in complete medium (RPMI 1640 + GlutaMAX™ with 10 % (FCS), 1 % sodium pyruvat, 1 % non-essential amino acid and 1 % penicillin streptomycin). Cells were counted and viability was measured by Trypan Blue (#1753468, Life technologies) staining on a CountessTM automated cell counter (InvitrogenTM). 5, 10, 20 or 50 million PBMCs in complete medium were mixed 1:1 with freezing solution (FCS + 20 % dimethyl sulfoxide). The tubes were then put into a Mr Frosty freezing box containing isopropanol and frozen at -80°C overnight before transfer to -150 °C the next day.

3.1.3 Thawing of PBMCs

PBMCs were thawed in a 37°C water bath and washed with 10 ml warm complete medium (first, 1 ml complete medium was added slowly drop by drop, before the remaining 10 mL were added). The samples were then centrifuged at 300 g, 4°C for 8 minutes. Samples with red pellets (i.e. indicating the presence of erythrocytes) were lysed as previously described. The supernatant was removed and the cells were resuspended in 2 mL complete medium. Next, the cells were counted and viability was measured. The cells were rested overnight in a humidified incubator with 5 % CO2.

3.1.4 Handling of HIV infected cells

Since HIV is a contagious virus with no curative treatment it has to be handled with extra care. Gloves were used at all times when in contact with HIV+ material. Protective glasses and special plastic coated lab coats intended for working with virus and other infectious material were used. Live HIV+ cells were always handled in a laminar flow cabinet (LAF bench) in a biosafety level 2 lab. Pipette tips, tubes and other equipment that were in direct contact with the HIV+ cells were put in bottles with Rely+On™ Virkon (Du Pont) and

discarded in bio-hazard waste. The Virkon solution was made with 1 liter of dH_2O and 30 grams Virkon powder. Also the biological material was inactivated with Virkon. For the waste box connected to the flow cytometer, Sodium hypochlorite 14 % C12 in aqueous solution (VWR Chemicals) was added to the waste before and after use and left for 24 hours before it was discarded.

3.2 Analysis by flow cytometry

An LSR Fortessa flow cytometer (BD Bioscience) equipped with 4 lasers and the BD FACSDiva™ software was used for analysis. Before analysing the samples, a compensation matrix was set. Fluorochromes emit light over a range of wavelengths which, even in the presence of filters, can result in a false signal being recorded in detectors meant for other fluorochromes. This "spillover" needs to be corrected for. By compensating, a mathematical process subtracts false fluorescence from a detector in order to allow only the true fluorescence to be recorded as an event. Compensation was performed by mixing the individual antibodies (abs) with UltraComp eBeads (#01-2222-42, Thermo Fisher Scientific). To calculate the spillover from the live/dead marker 7AAD, a mix of live and dead cells were used.

3.2.1 Titration of Abs

Abs used for surface staining (Table 2) and intracellular staining of cytokines (Table 3) were purchased from either BD Biosciences or BioLegend. The abs were titrated to find the optimal concentration. Since so many Abs against different cell surface markers were to be used at the same time it was important to find the optimal concentration, so the signal would be maximized and noise reduced.

For the titration, the same protocol that would be applied for the future experiments were used; Ab to be used for staining of surface markers were titrated on live PBMCs, whereas Ab for intracellular staining were titrated on fixated and permeabilized cells. Time and temperatures, as well as volumes were kept constant and similar to the future experiments. For most Ab, four different concentrations in approximately 2 fold dilutions series were tested.

3.2.2 Staining of cell surface markers

In total, 52 patient samples were analysed. 11 patient samples + 1 sample from a healthy blood donor (serving as an internal standard) were analysed at the same time. The surface markers of interest were CD3, CD4, CD45RA, CD27, CD57, CD38, HLA-DR, PD-1, Lag-3, Tim-3, CD160, TIGIT, KLRG1 and integrin β7. 7AAD was included to exclude dead cells.

The cell surface receptors CD3 and CD4 are not exhaustion markers, but were included in the panel to identify T cells. All T cells should express this marker; hence, CD3 serves as a marker to identify T cells. To also stain with CD4 is important to be able to distinguish between CD4 and CD8 T cells.

Each sample was distributed in four wells in a 96 V-bottom plate: one well for staining cells with all the 15 markers and three other wells for FMO (fluorescence minus one) controls. It would have been optimal to have FMO controls for all the Abs in the panel, but because of limited patient material and many markers included in the panel, it was not possible to have all the ideal FMO controls. The cell numbers in the patient samples collected for this study varied from 1 million to 10 million cells per sample. The FMO control contains all the fluorochromes in the panel, except for the one being measured. In this case, fluorescence minus 3 was used because in each control, 3 fluorochromes were excluded. Based on spillover data from the compensation matrix, HLA-DR, Tim-3 and CD160 were excluded together, CD38, PD-1 and Lag-3 together and Integrin β7, TIGIT and KLRG1 together. "True" FMO controls were included for CD57 and 7AAD, but only for the internal standard. The cell surface markers CD3, CD4, CD27 and CD45RA were easily gated for without FMO control, so these cell surface markers were analysed without FMO controls. 500 000 cells per well were stained in a total of 50 µl.

Table 2. Abs used for detection of surface markers.

Antigen	Fluorochrome	Clone	Company (material number)	Amount/ 50 µl
CD3	PerCP	UCHT1	BioLegend (#300428)	0.5 µl
CD4	AX700	SK3	BioLegend (#344621)	0.4 µl
CD45RA	APC-H7	Hl100	BD Biosciences (#560674)	0.5 µl
CD27	BV510	L128	BD Biosciences (#563090)	0.25 µl
CD38	AX488	H1T2	BioLegend (#303512)	1.25 µl
HLA-DR	BV711	G46-6	BD Biosciences (#563696)	1.25 µl
PD-1 (CD279)	BV785	EH 12.2H7	BioLegend (#329930)	2.5 µl
Lag-3 (CD223)	PE	11C3C65	BioLegend (#369305)	2.5 µl
Tim-3	BV650	F38-3E2	BioLegend (#344621)	2.5 µl
CD160	PE-Cy7	BY55	BioLegend (#341212)	1.5 µl
TIGIT	BV421	A15153G	BioLegend (#372709)	1.25 µl
CD57	PE/Dazzle 594	HNK-1	BioLegend (#359619)	0.25 µl
KLRG1	BV605	2F1/KLRG1	BioLegend (#138419)	0.5 µl
Integrin β7	APC	F1B504	BioLegend (#321208)	1 µl
7-AAD (Live-dead marker)			BD Biosciences (#51-2359KC)	2 µl

Cells were stained for 30 minutes, protected from light at room temperature. Cells were then washed twice with PBS + 2 % FCS, and resuspended in 200 µl PBS + 2 % FCS. 7AAD was added a minimum of 5 minutes before the cells were analysed on the flow cytometer.

3.2.3 Intracellular staining of cytokines

Cells were fixated (Human FoxP3 buffer A, BD Pharmingen #560098) and permeabilized (Human FoxP3 buffer C, BD Pharmingen #650098) before stained with the appropriate Abs. All cells were stained with Abs against the surface markers CD3 and CD4, as well as Abs against the intracellular cytokines IFNγ, TNF, IL-2, and TGF-β. Due to limited patient material, technical controls like FMOs were not included. Instead, unstimulated cells served

as both biological controls as well as to guide the setting of gates for cytokine producing cells. After staining for 30 minutes at room temperature in the dark, the cells were washed twice and resuspended in 200 µl PBS + 2 % FCS and analysed on the flow cytometer.

Table 3. Abs used for intracellular staining.

Antigen	Fluorochrome	Clone	Company (material number)	Amount/50 µl
CD3	Pacific blue	UCHT1	BD Biosciences (#558117)	0.25 µl
CD4	PerCP		BD Biosciences (#550631)	1 µl
IFNγ	AX488	45.B3	BioLegend (#502525)	0.5 µl
TNF	PE/Cy7	Mab11	BioLegend (#502929)	0.5 µl
IL-2	BV785	MQ1-17H12	BioLegend (#500348)	0.5 µl
TGFβ1	PE	TW4-3F8	BioLegend (#349603)	1.5 µl
PD-1 (CD279)	PE	EH 12.1	BD Biosciences (#560795)	7.5 µl

3.2.4 Gating strategy

Flow cytometry data were analysed using the FlowJo™10 (Tree Star, Inc) software. The analysis of the patient material was blinded to reduce bias. A hierarchical gating strategy was applied in order to investigate the desired cells (Fig. 5). First, side scatter area was plotted against time. This plot will show the time the collected events were recorded in the flow cytometer. If something unexpected happened during analysis (e.g. clogs or air in the fluid stream) this can be gated out. Next, side scatter area against 7AAD was used to gate away all the dead cells. Lymphocytes were selected by plotting side scatter area against forward scatter area. Cells that may have clustered together and formed doublets was gated away by plotting forward scatter width against forward scatter height. CD4+ and CD8+ T cell populations were identified by plotting CD3 against CD4. The CD3+ CD4+ and the CD3+ CD4- (assumed to be CD8+ T cells) populations were further analysed for the various surface markers or intracellular cytokines (SFig.1).

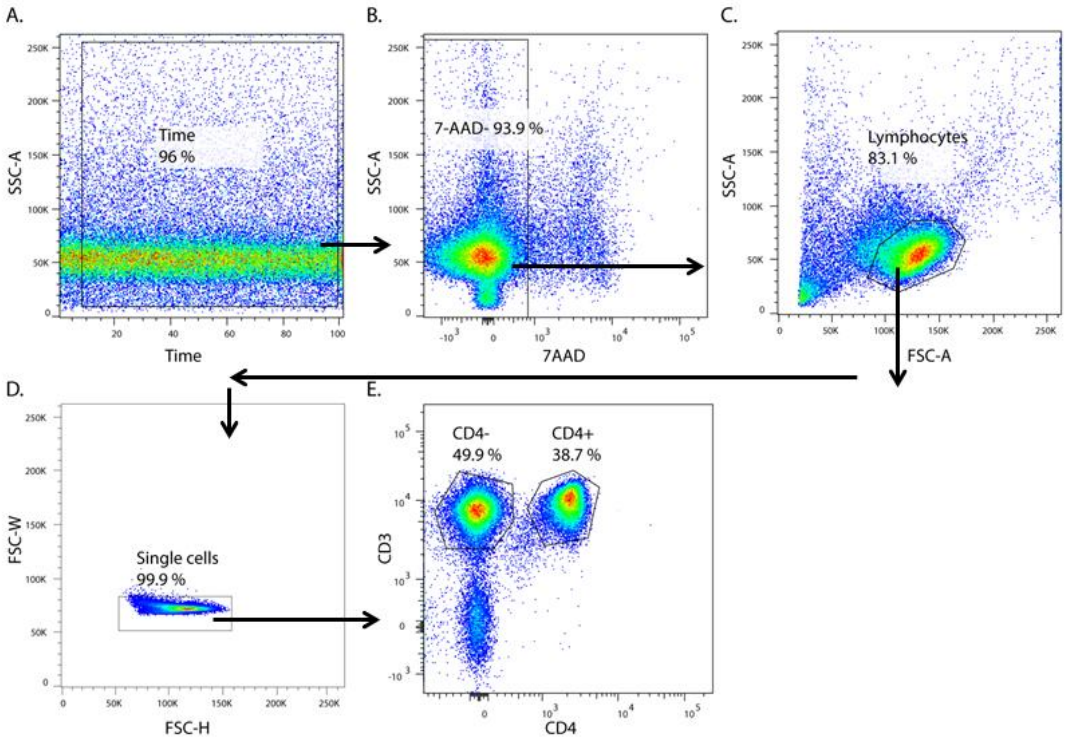


Figure 5. Gating strategy. *The gating starts in the upper left plot and follows the direction of the arrows. Unexpected events and dead cells are first gated out (A-B). Lymphocytes are selected based on size and granularity (C) and single cells are gated for in order to exclude doublets (D). CD3 against CD4 was used to gate for the CD4+ (CD3+ CD4+) and CD8+ (CD3+ CD4-) T cell populations (E). The cell surface markers were further analysed inside the CD4 and the CD8 populations.*

3.2.5 Statistical analysis

Statistical analyses were performed in GraphPad Prism 7.02. This program was used to analyse the frequency distribution data from the experiments. Graphs with comparison data were made with a scatter dot plot style and P-values were calculated with the nonparametric Mann-Whitney test. Correlation was calculated with the nonparametric Spearman test.

3.3 Functional in vitro T cell stimulation assays

3.3.1 Stimulation of T cells

To stimulate T cells, beads coupled with anti-CD3 and anti-CD28, and in some instances anti-CD2, were used. Anti-CD3 and anti-CD28/CD2 will give the signals needed for T cell activation. In general and if not otherwise stated, 200 000 cells were stimulated with a 3:1 bead-to-cell ratio in 200 µl medium. The cells were stimulated for 6 hours at 37 ºC together with 10 µg/mL Brefeldin A (Sigma-Aldrich). By adding Brefeldin A to the cells, protein transport from the endoplasmic reticulum to the Golgi apparatus will be inhibited. By inhibiting this transport, cytokines are trapped inside the cells. This makes it easier to stain for the appropriate cytokines later. The stimulation was stopped by fixing the cells as described in section 3.2.3.All samples were also analysed for surface expression of PD-1, as this would be important for the results of the stimulation/inhibition assays regarding PD-1 (see section 3.3.2).

3.3.2 Engagement of PD-1

In preliminary experiments, 1 µg/ml biotinylated PD-L1-IgG2a obtained from Dr. Gordon Freeman, was coupled to MACSi Bead™ particles together with 10 and 5 µg/ml of biotinylated anti-CD3/CD28, respectively (#130-091-441, MACS Miltenyi Biotech). As the expected results did not occur, another procedure with M-450 Epoxy Dynabeads™ (#14011, Thermo Fisher Scientific) was established. M-450 Epoxy Dynabeads are superparamagnetic, monosized polymer beads that are coated in glycidyl ether (Epoxy) groups. This coating makes the beads bind covalently to amino- and sulphydryl groups in e.g. proteins. Hence, Abs and other proteins can be bound to the beads without a secondary molecule like biotin. The Dynabeads were coupled with LEAF purified anti-human CD3 antibody (#317304, BioLegend) and LEAF purified anti-human CD28 antibody (#302914, BioLegend) in different concentrations (see Table 4), as well as recombinant human PD-L1/B7-H1 Fc Chimera (#156-B7-100, R&DSystems) or an isotype control human IgG1 Kappa-LEAF (#0151K-14, Southern Biotech) (Fig. 6).

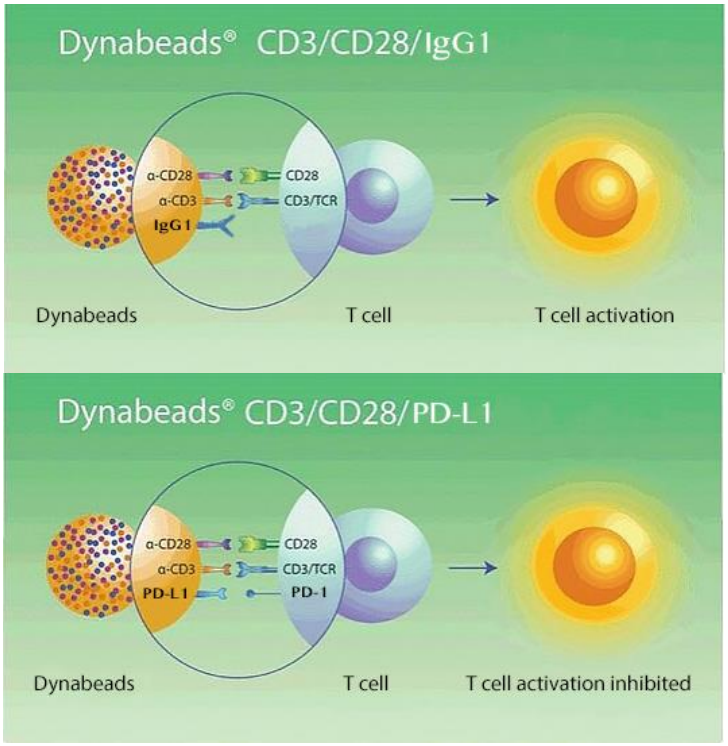


Figure 6. Schematic overview of M-450 Epoxy Dynabeads coupled with anti-CD3, anti-CD28 and IgG1/PD-L1. *Cells stimulated with Dynabeads coupled with anti-CD3, anti-CD28 and IgG1 will give the signals needed to activate T cells (upper image)). Cells stimulated with anti-CD3, anti-CD28 and PD-L1 will result in interaction between PD-1 and PD-L1 and inhibit T cell signaling and activation (lower image). Modified from Thermo Fisher Scientific webpage [151].*

Table 4. Composition of Abs coupled to the Dynabeads

Anti-CD3	**Anti-CD28**	**IgG1/PD-L1**
20 % (8µg)	20 % (8 µg)	60 % (24 µg)
10 % (4 µg)	10 % (4 µg)	80 % (32 µg)

Before coupling, $4x10^8$ beads were washed with buffer 1 (0,1M sodium-phosphate, pH 7,4). After 1 minute in a magnet, the supernatant was discarded and the beads were resuspended in 450 µl buffer 1. The beads were divided into separate tubes, containing 80 000 000 beads per tube. Anti-CD3, anti-CD28, IgG1 and PD-L1 were added to the respective tubes. The beads were then incubated for 72 hours under constant rotation. Next, the beads were washed three times with buffer 2 (PBS with 0,1 % BSA and 2 mM EDTA, pH 7,4). To get the optimal concentration of $4x10^8$ beads/ml, the beads were resuspended in 200 µl buffer 2.

The lab has a long experience in stimulating T cells with beads. Assays are regularly performed with 10 µg/ml biotinylated anti-CD3/CD28/CD2 coupled to MACSi Bead^{TM} particles. To be sure that the M-450 Epoxy Dynabeads worked, such MACSi beads were used as positive controls in preliminary stimulation experiments. The MACSi bead-to-cell ratio in the experiments was 1:2. PMA + ionomycin were also used as positive control in some experiments. PMA is an organic compound which diffuses through the cell membrane into the cytoplasm, where it directly activates protein kinase C which induces an unspecific TCR stimulation. Ionomycin, which is a calcium ionophore, triggers the release of calcium which also is needed for further signaling.

3.3.3 Time, concentration and bead-to-cell ratio

Several experiments were performed in order to decide which assay conditions that best displayed the effect of PD-1 ligation. First, the optimal amount of anti-CD3/CD28 and PD-L1 coupled to the beads was determined. Next, the stimulation time was investigated.

Activated T cells can produce large amounts of cytokines. Depending on stimulation time, production of cytokines can vary. To analyse the production of IFNγ, TNF, IL-2, IL-10 and TGF-β1, 6 hours and 24 hours of stimulation were tested. The experiment was tested on PBMCs from both healthy blood donors and HIV+ patients. Two 96 well U-bottom plates were prepared with wells containing 200 000 cells in 200 µl complete medium. M-450 Epoxy Dynabeads coupled with 20 % anti-CD3 and 20 % CD28 and 60 % IgG1 or PD-L1 was added in a 3:1 bead-to-cell ratio.

Also, the bead-to-cell ratio was titrated. Different ratios were tried out in the different experiments on both PBMCs from healthy blood donors and from HIV+ patients. The bead-to-cell ratios were 10:1, 6:1, 3:1, 1:1 and 1:3, and the stimulation time was 6 hours.

3.3.4 Blocking of PD-1/PD-L1 interactions

In order to restore cytokine production, a monoclonal blocking Ab against PD-1 (PD-1 (CD279) Clone PD.1.3.1.3) was tested. The cells were preincubated with anti-PD1 for 1 hour before addition of beads to the cell cultures.

After 6 hours of stimulation, the cells were fixed, permeabilized and stained using the same procedure as explained earlier. To decide the amount of anti-PD1 necessary to abolish PD1-

PD-L1 interaction and to boost cytokine production, anti-PD1 was titrated. The anti-PD1 titration series used were 0.1 μg/mL, 1 μg/mL, 10 μg/mL and 50 μg/mL.

4 Results

The research in this Thesis consists of two parts where the first part is a phenotypical study where cell surface markers involved in differentiation and exhaustion on T cells were analysed. The expression levels of these cell surface markers were then compared between the INR, IR and HC groups. The second part of this Thesis deals with establishing a procedure for functional studies in order to understand the effect of exhaustion and block it.

4.1 Comparison of T cell phenotype in INRs, IRs and HCs

4.1.1 Study Participants and Baseline Characteristics

To investigate cell surface markers in HIV+ patients, 19 INR patients, 17 IR patients and 16 HC were included. Table 5 shows the cohort characteristics. All study participants were matched on age. The INR and IR patients were also matched on nadir CD4 and CD8 count. The median CD4 count in the INR group was 334 cells/µl compared with 741 cells/µl in the IR group ($P < 0.0001$), hence INR patients also had a significant lower CD4/CD8 ratio ($P < 0.0001$). There were also significant differences in the duration of disease ($P = 0.0236$) and continuous ART use, with IRs having the longest infection and ART treatment ($P = 0.0110$) (Table 5). Participants were excluded from the study if they were hepatitis C or B positive, had comorbidity of inflammatory bowel disease, coeliac disease or malnutrition, or if they had liver or heart failure.

Table 5. Characteristics of the study cohorts at inclusion.

Total Study Population	INR (n = 19)	IR (n = 17)	HC (n = 16)	INR vs IR, P
Age	47.8 (38.4 – 65.8)	52.2 (37.1 – 64.3)	54.4 (33.2 – 64.9)	NS
Length of infection (years)	9.5 (4.5 – 24.1)	20.5 (5.6 – 32.0)		0.0236
Length of treatment	9.9 (4.2 – 19.9)	16.7 (5.3 – 22.9)		0.0110
CD4 count nadir, cells/µl	115 (8 – 234)	102 (10 – 240)		NS
CD4 count, cells/µl	334 (168 – 466)	741.5 (427 – 1083)		< 0.0001
CD8 count, cells/µl	749 (350 – 2239)	758 (439 – 1444)		NS
CD4/CD8	0.48 (0.18 – 0.86)	1 (0.48 – 1.86)		< 0.0001
Viral load in plasma	< 20 copies/mL	< 20 copies/mL		NS

INR, immunological nonresponders; IR, immunological responders; HC, healthy controls; NS, not significant

4.1.2 INR patients had decreased CD4+ T cell count compared to IRs

T cells can be distinguished from other immune cells by the expression of the cell surface markers CD3, CD4 and CD8. By taking advantage of this, the presence of T cells in PBMCs was studied by staining with Abs and analyzing by flow cytometry.

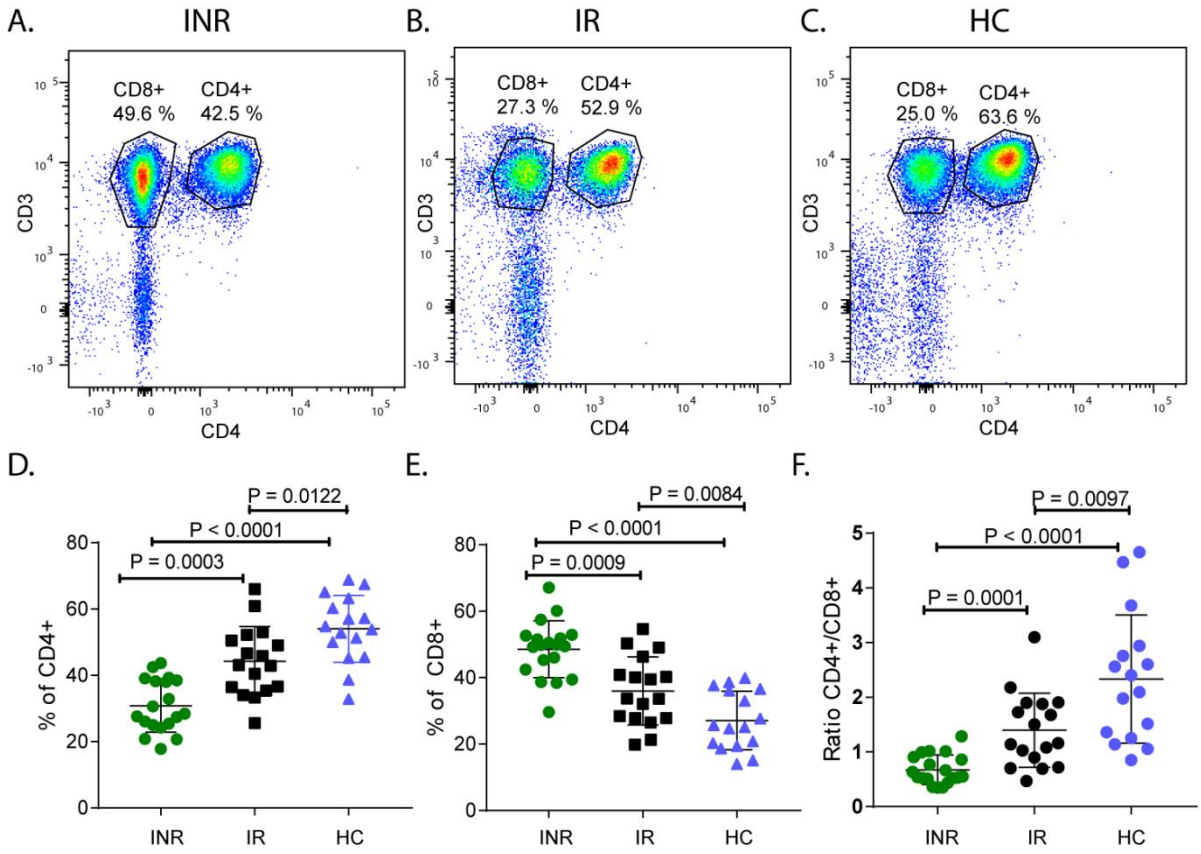


Figure 7. Comparison of the CD4 and CD8 T cell distribution. *PBMCs were analysed by staining with anti-CD3 and anti-CD4 Abs. CD8+ T cells were defined as CD3+ CD4-. A-C) Representative flow cytometry images of T cells from an INR patient (A), IR patient (B), and HC (C). D-F) Comparison of the percentages of CD4+ T cells (D), CD8+ T cells (E) and the CD4/CD8 ratio (F). The p values were calculated with the non-parametric Mann Whitney U-test. Lines indicate the mean ± SD.*

INR patients had decreased percentages of CD4+ T cells compared with IR ($P = 0.0003$) and HC ($P < 0.0001$) (Fig. 7). The low levels of CD4+ T cells in INRs result in higher percentages of CD8+ T cells, which was significantly increased compared with IR ($P = 0.0009$) and the HC group ($P < 0.0001$). As a result of this, the CD4/CD8 ratio was lower in the INR group than in the IR ($P = 0.0001$) and HC ($P < 0.0001$) groups (Fig. 7F). Interestingly, even though IR had higher CD4/CD8 ratios than the INR patients, they did not reach the levels of HC (P =

0.0097). The results from the flow cytometry analysis reflect the cell count numbers (listed in Table 5) obtained by the Department of Immunology.

4.1.3 Differentiation status of T cells were similar between INRs, IRs and HCs

To investigate whether INR patients had a different T cell differentiation status than IR and HC, the cells were stained with anti-CD45RA and anti-CD27 Abs (Fig. 8A).

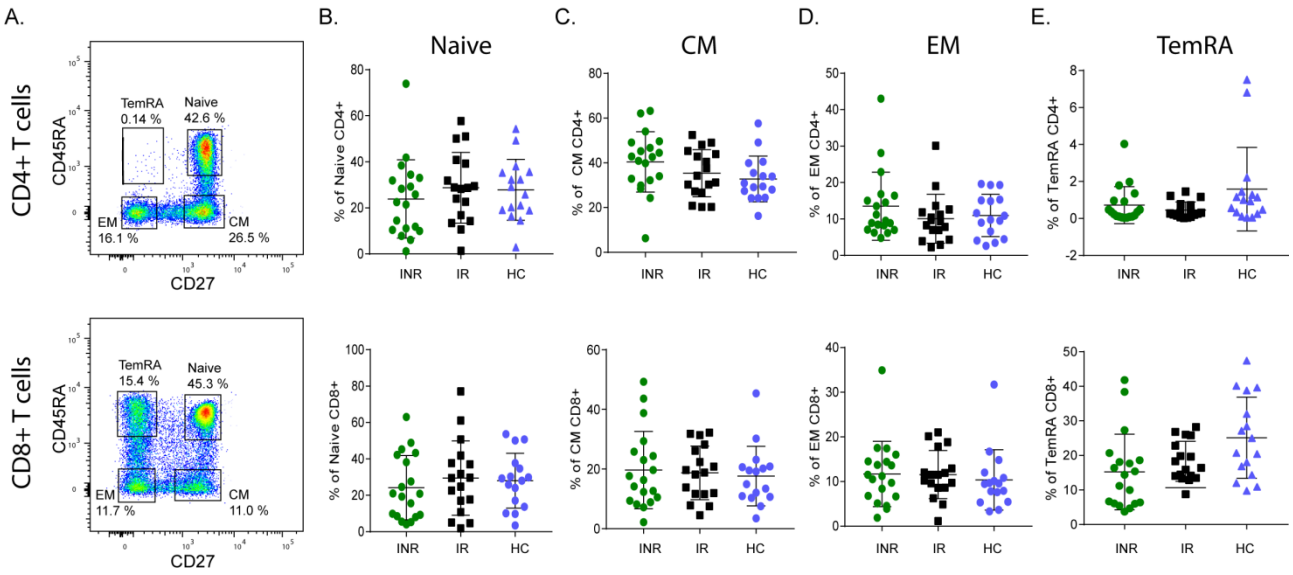


Figure 8. Differentiation status of T cells in INRs, IRs and HCs. *PBMCs were stained with anti-CD45RA and anti-CD27 Abs to distinguish T cells at different differentiation levels. The upper panels show CD4+ T cells and the lower panels show CD8+ T cells. (A) Representative flow cytometry image of CD4+ T cells (top) and CD8+ T cells (bottom) from an INR patient. (B) Comparison of the percentage of naive (CD45RA+ CD27+) CD4+ and CD8+ T cells. (C) The percentages of CM (CD45RA- CD27+) CD4+ and CD8+ T cells. (D) Percentages of EM (CD45RA- CD27-) CD4+ and CD8+ T cells. (E) Percentages of TemRA (CD45RA+ CD27-) CD4+ and CD8+ T cells. Lines indicate the mean ± SD.*

The percentages of CD4+ and CD8+ T cell subsets with differentiation status, naive (CD45RA+ CD27+), CM (CD45RA- CD27+), and EM (CD45RA- CD27-), and TemRA (CD45RA+ CD27-) were similar between the groups (Fig. 8). The INR group showed a tendency of lower Naive/EM CD4 ratio compared to IR (P = 0.0998), but the Naive/CM ratio were similar between the groups (SFig. 2).

4.1.4 Similar levels of activated T cells between INRs, IRs and HCs

To investigate whether INR patients had a more activated phenotype compared to IR and HC patients, the cells were stained with anti-HLA-DR and anti-CD38 Abs (Fig. 9A). Activated T cells can be identified by the expression of CD38 alone or in combination with HLA-DR.

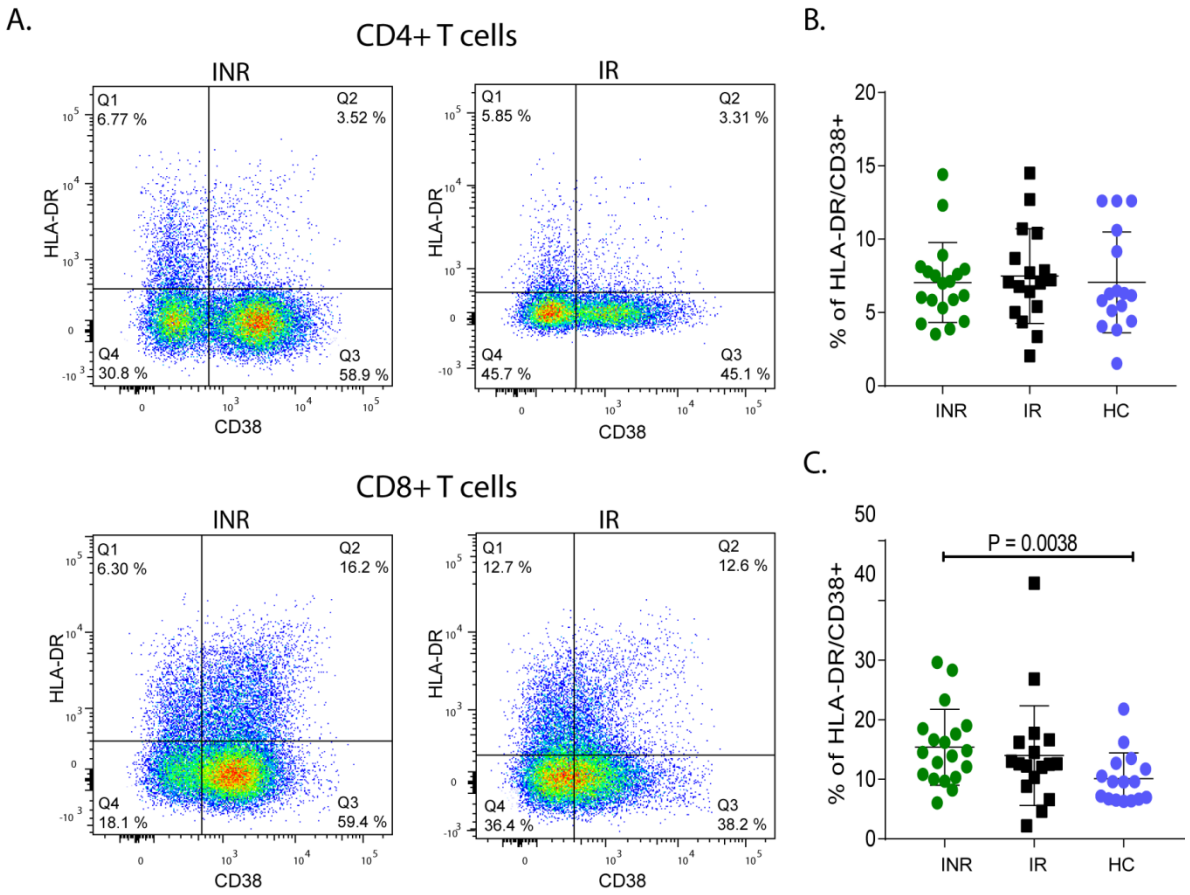


Figure 9. Activation status of T cells in INRs, IRs, and HCs. *The quadrant gates were set based on the FMOs for HLA-DR and CD38. T cells determined as HLA-DR+ and CD38+ are shown in Q2. A) Representative flow cytometry images of CD4+ and CD8+ T cells from an INR patient (left) and an IR patient (right). B) Comparison of the percentages of HLA-DR+ CD38+ CD4+ T cells for the three groups. C) Comparison of HLA-DR+ CD38+ CD8+ T cells from the three groups. The p value is indicated where significant (Mann-Whitney U-test). Lines indicate the mean ± SD.*

There were no differences in the fractions of CD38+ HLA-DR+ double positive T cells between the INR and IR groups (Fig. 9B and C). However, the INR group had higher percentages of CD38+ HLA-DR+ CD8+ T cells compared to the HC group (P = 0.0038) (Fig. 9C). When looking at CD38 expression alone, no significant differences in percentages of

CD38+ CD4+ or CD8+ T cells were found (SFig. 3). Analysis measured by higher staining intensity (MFI) were also done to see if the INR group expressed higher amounts of CD38 T cells than the two other groups. This analysis also showed similar levels of expression between the groups (SFig. 4).

4.1.5 INR patients had higher levels of T cells expressing PD-1 and TIGIT

The cells were stained with Abs against PD-1, TIGIT, Lag-3, Tim-3 and CD160 to investigate a potential exhaustion state of the T cells in INRs, IRs and HCs.

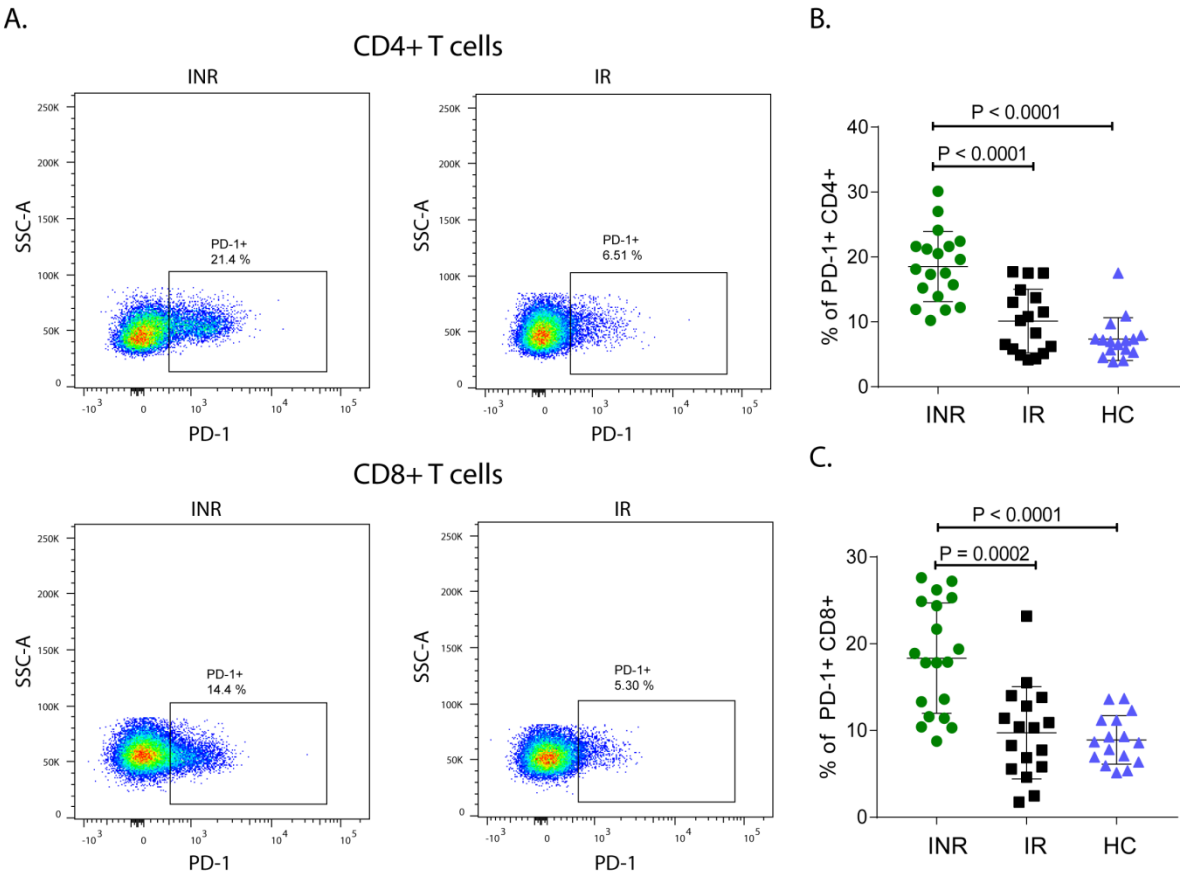


Figure 10. PD-1 expression on T cells. *PBMCs were analysed by staining with anti-PD-1. Gates were set based on the FMO control for PD-1. A) Representative flow cytometry images of CD4+ T cells (upper panel) and CD8+ T cells (lower panel) from an INR patient (left) and an IR patient (right). B) Comparison of the percentages of PD-1+ CD4+ T cells for the three groups. C) Comparison of PD-1+ CD8+ T cells from the three groups. P values are indicated (Mann-Whitney U-test). Lines indicate the mean ± SD.*

Patients in the INR group had higher percentages of PD-1+ CD4+ T cells than the IR patients ($P < 0.0001$) and the HC group ($P < 0.0001$) (Fig. 10B). The same results were seen for PD-

1+ CD8+ T cells where INR patients had significantly increased expression of PD-1 than IR patients (P = 0.002) and the HC group (P < 0.0001) (Fig. 10C).

TIGIT, a second exhaustion marker, was also found to be upregulated in INR patients in CD4+ T cells compared with the IR (P = 0.0007) and HC group (P < 0.0001) (Fig. 11B). INRs CD8+ T cells also expressed higher fraction of TIGIT+ T cells than IR (P = 0.0499) and the HC group (P = 0.0001) (Fig. 11C). The IR group also displayed a significant increase of TIGIT+ CD4+ T cells compared to HC patients (P = 0.0247) (Fig. 11B). Noteworthy, the expression of neither PD-1 nor TIGIT was differentially expressed between the groups with regards to fluorescence intensity (data not shown).

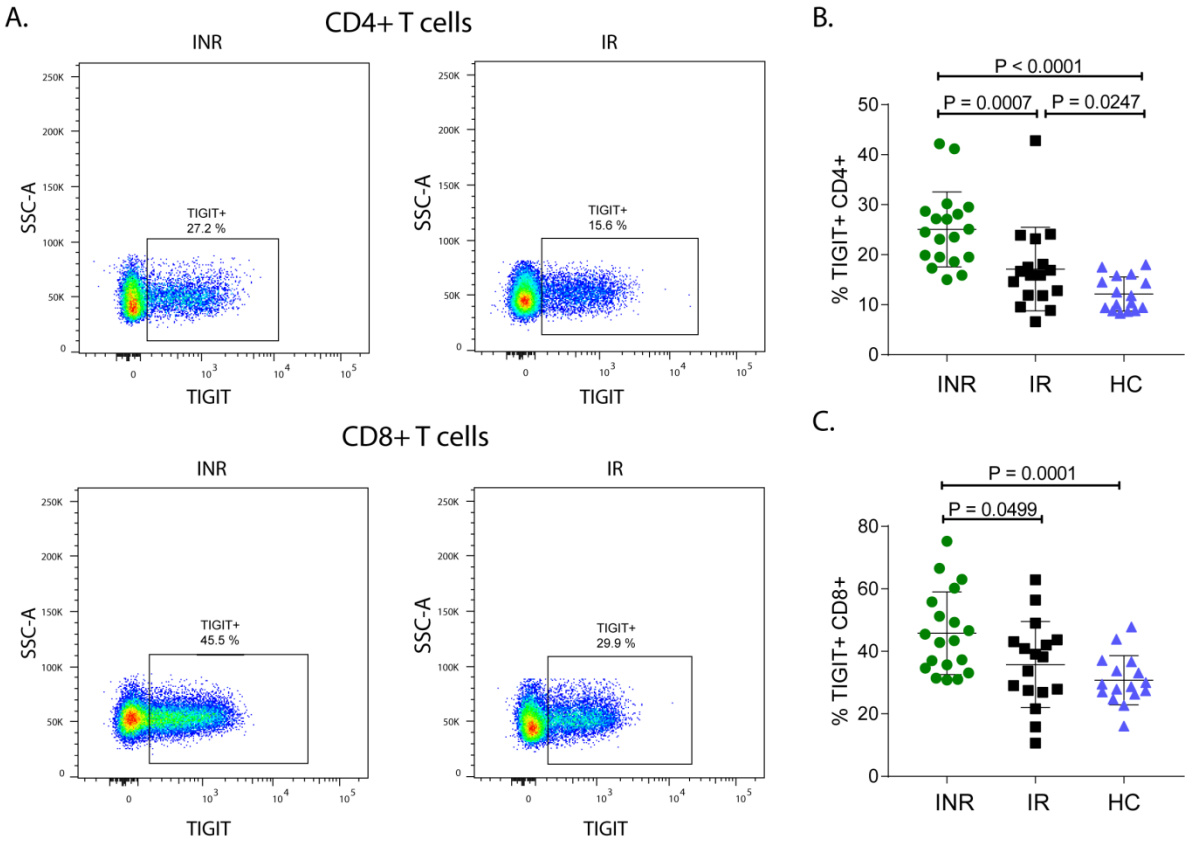


Figure 11. T cells expressing TIGIT in INRs, IRs and HCs. *PBMCs were analysed by staining with anti-TIGIT. Gates were set based on the FMO control for TIGIT. A) Representative flow cytometry images of CD4+ T cells (upper panel) and CD8+ T cells (lower panel) from an INR patient (left) and an IR patient (right). B) Comparison of the percentages of TIGIT+ CD4+ T cells for the three groups. C) Comparison of TIGIT+ CD8+ T cells from the three groups. P values are indicated (Mann-Whitney U-test). Lines indicate the mean ± SD.*

Since INR patients had more T cells expressing PD-1 and TIGIT compared to the other groups, further analysis was done to see if the INR patients also showed higher levels of T cells expressing both PD-1 and TIGIT (Fig. 12).

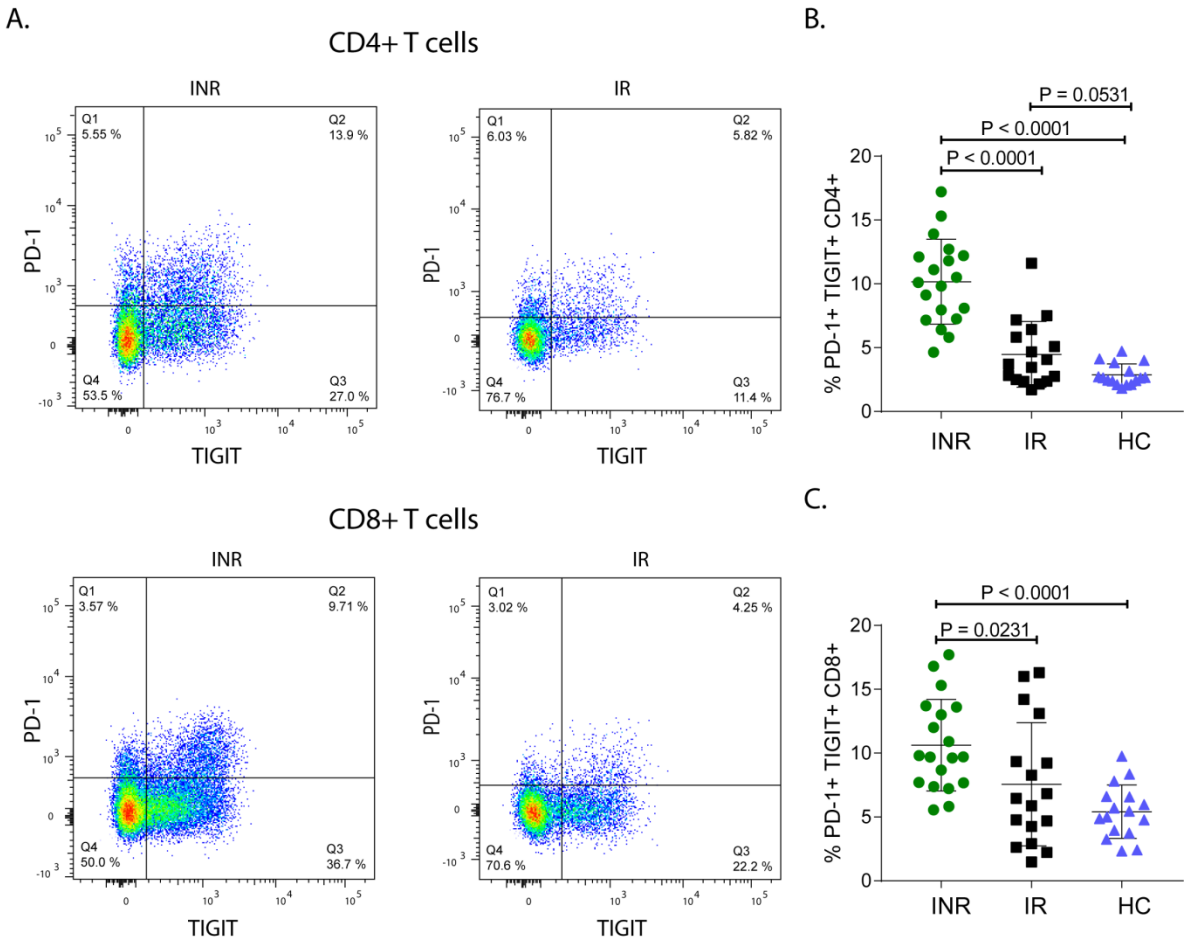


Figure 12. T cells co-expressing PD-1 and TIGIT in INRs, IRs and HCs. *PBMCs were stained with anti-PD-1 and anti-TIGIT Abs in order to compare PD-1+ TIGIT+ T cells between the groups. The quadrant gates were set based on the FMOs for PD-1 and TIGIT. A) Representative flow cytometry images of CD4+ T cells (upper panel) and CD8+ T cells (lower panel) from an INR patient (left) and an IR patient (right). B) Comparison of the percentages of PD-1+ TIGIT+ CD4+ T cells for the three groups. C) Comparison of PD-1+ TIGIT+ CD8+ T cells from the three groups. The p value is indicated (Mann-Whitney test). Lines indicate the mean ± SD.*

INR patients showed relative increased levels of PD-1 and TIGIT double positive CD4+ and CD8+ T cells compared to IR patients and the HC group (Fig. 12B and C). No significant differences were found between the IR and HC groups, neither for the CD4 or the CD8 T cells. (Fig. 12B and C).

Next, we wanted to study if the expression of exhaustion markers had any correlation with CD4+ T cell numbers. The amount of double positive PD-1+ TIGIT+ T cells correlated negatively with the CD4 T cell percentages looking at all groups combined ($r = -0.6445$, $P < 0.0001$) (Fig. 13A). There was also a negative correlation between single positive (PD-1 or TIGIT+) CD4+ T cells and CD4 percentages (data not shown). No negative correlation with CD4 counts was seen for PD-1+ TIGIT+ CD8+ T cells (Fig. 13B).

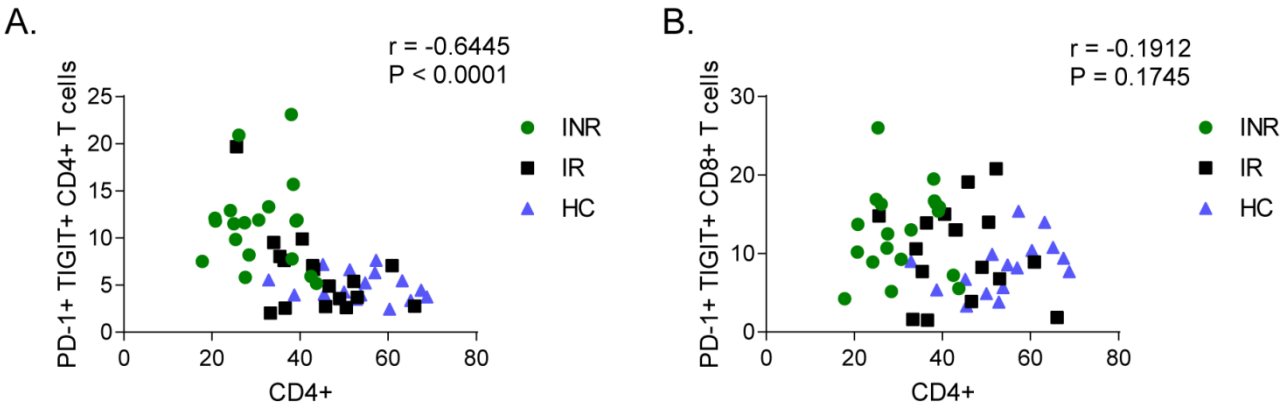


Figure 13. Correlation analysis of PD-1+ TIGIT+ T cells with CD4 percentages. *A) Correlation between PD-1+ TIGIT+ CD4+ T cells and CD4+ T cell relative levels (percentages). B) Correlation between PD-1+ TIGIT+ CD8+ T cells and CD4+ levels (percentages). R values were calculated with non-parametric Spearman correlation.*

Lag-3, Tim-3 and CD160 were not found to be differentially expressed in any of the groups (Fig. 14).

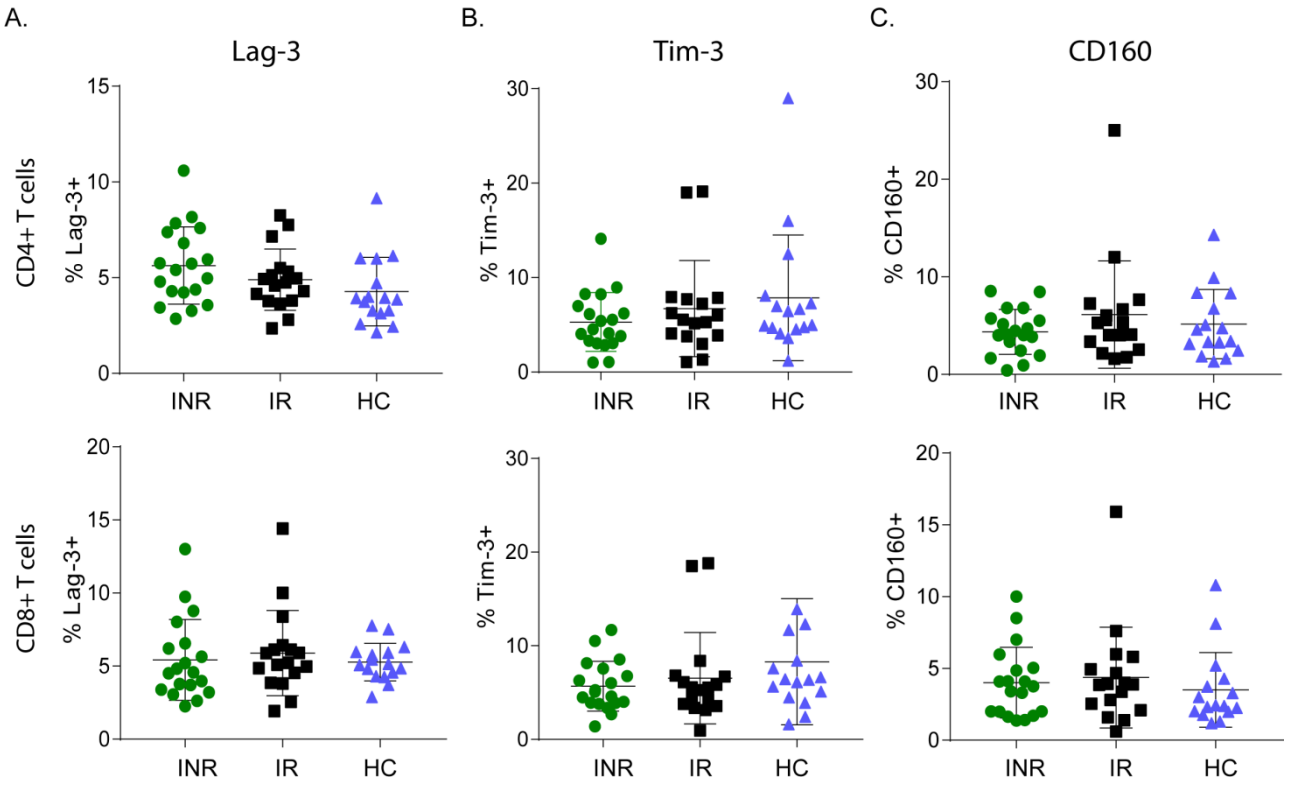


Figure 14. T cells expressing Lag-3, Tim-3 and CD160 in INRs, IRs and HCs. *PBMCs were stained with anti-Lag-3, Tim-3 and CD160 Abs in order to compare the expression levels between the groups. Percentages of CD4+ T cells (upper panels) and CD8+ T cells (lower panels) expressing, Lag-3 (A), Tim-3 (B), and CD160 (C). Lines indicate the mean ± SD.*

4.1.6 INRs showed similar expression of CD57 and KLRG1 compared to IRs and HCs

The two cell surface markers CD57 and KLRG1 were used in this study in order to explore T cell senescence. T cells expressing CD57 was gated in two different ways looking at both CD57+ T cells and CD57 high T cells (Fig. 15A). There were no differences in either CD57+ (Fig.15B) or CD57 high (Fig. 15C) expressing CD4+ or CD8+ T cells between the three groups.

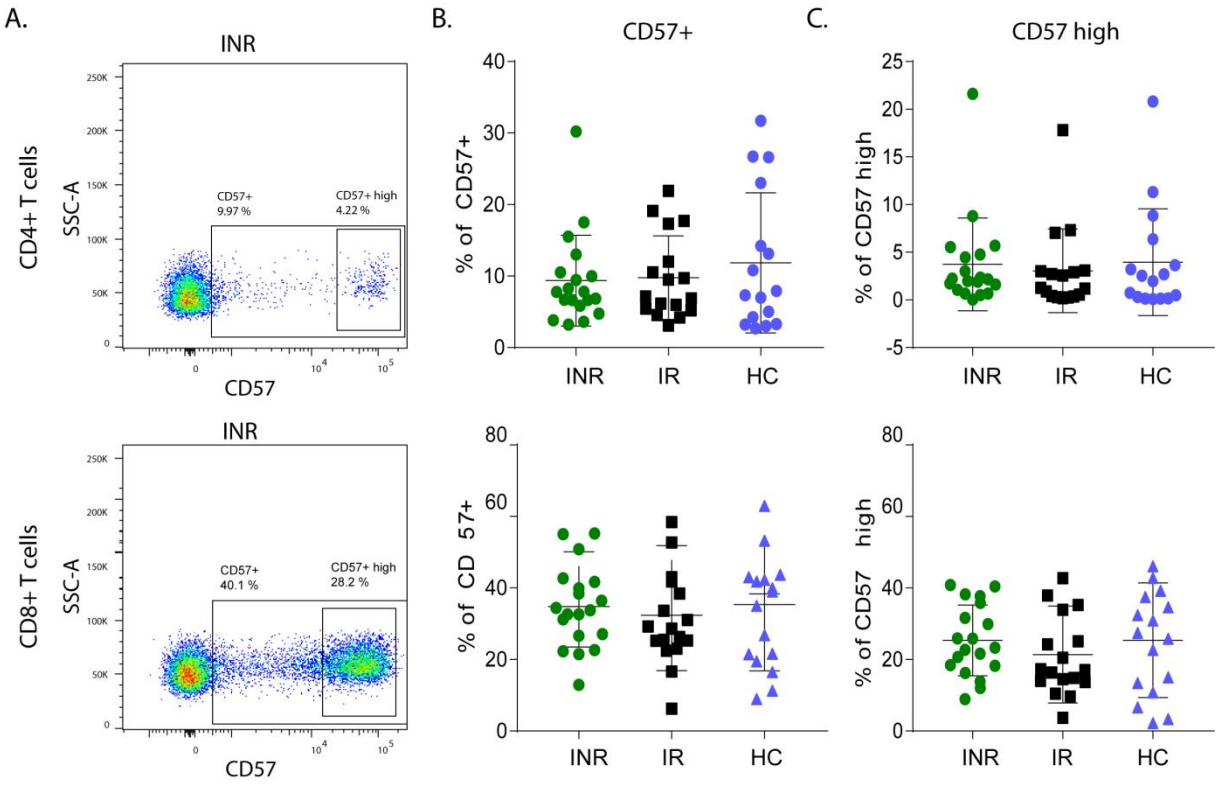


Figure 15. T cells expressing CD57 in INRs, IRs and HCs. *PBMCs were stained with anti-CD57 in order to distinguish the CD57+ T cells. Gates were set based on the FMO control for CD57. A) Representative flow cytometry images of CD4+ T cells (upper panel) and CD8+ T cells (lower panel) from an INR patient. B) Percentage of CD57+ CD4+ T cells (upper panel) and CD8+ T cells (lower panel). C) Percentages of CD4+ T cells (upper panel) and CD8+ T cells (lower panel) highly expressing CD57. Lines indicate the mean ± SD.*

The amount of CD57 high CD4+ and CD8+ T cells correlated negatively with the CD4 T cell percentages (r = -0.4073, P = 0.0027, and r = -0.3035, P = 0.0287, respectively) (Fig. 16A and B).

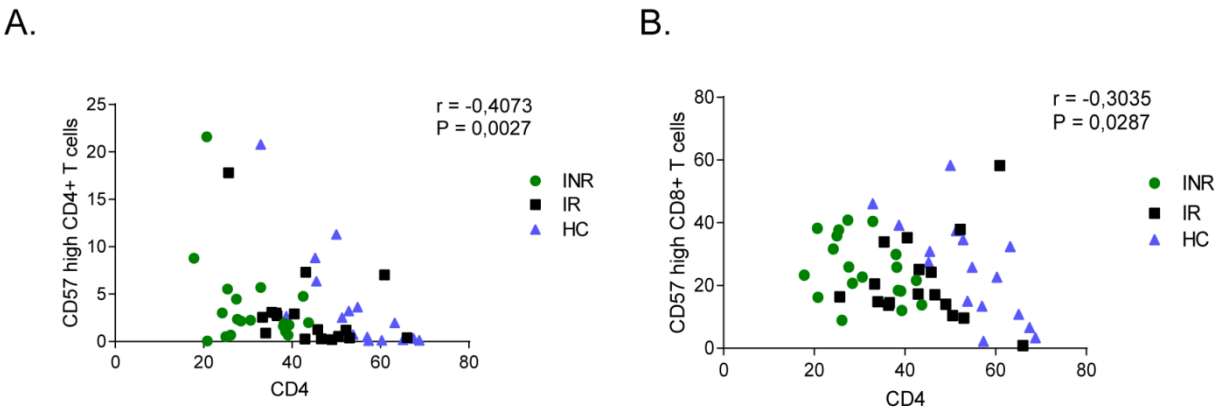


Figure 16. Correlation analysis of CD57 T cells with relative CD4 levels (percentages). *A) Correlation between CD57 high CD4+ T cells and CD4 levels. B) Correlation between CD57 high CD8+ T cells and CD4 levels. R values were calculated with non-parametric Spearman correlation.*

KLRG1 was also not found to be differentially expressed between the groups (Fig.17).

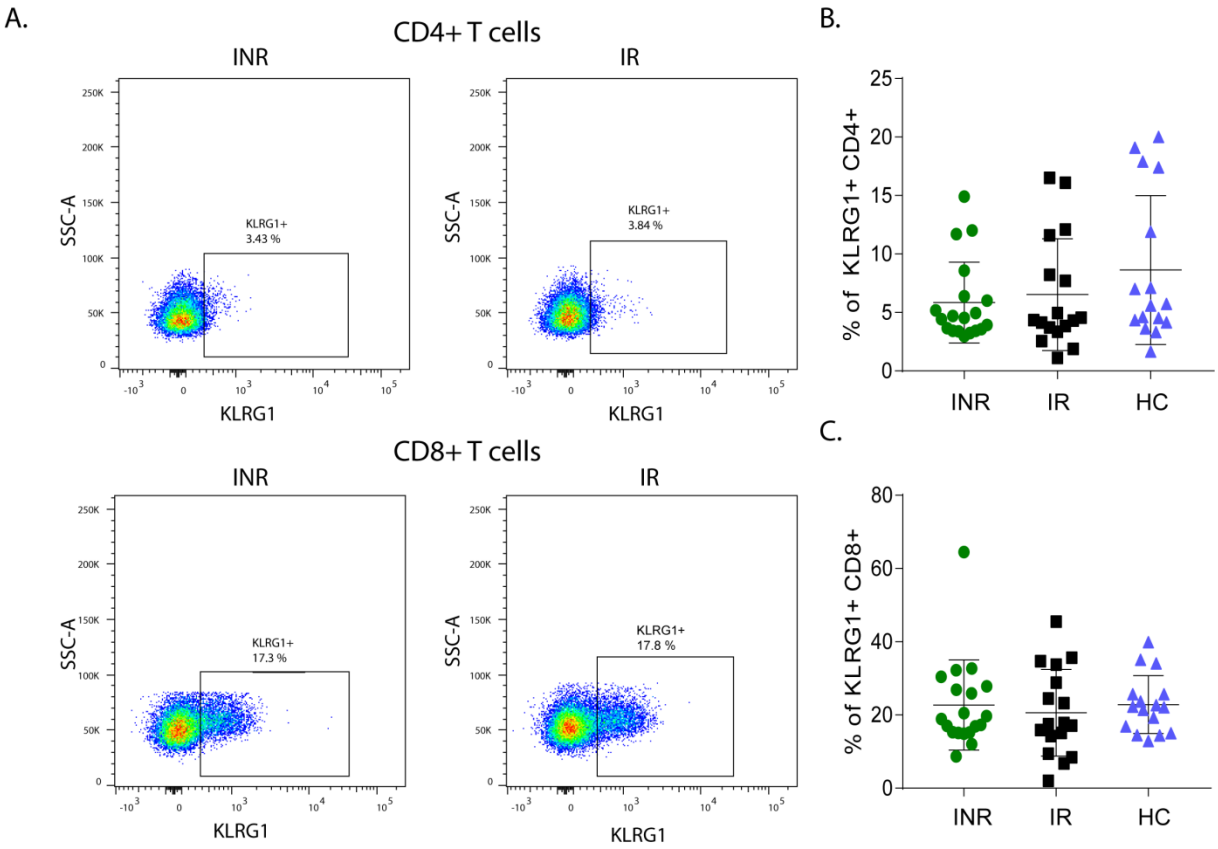


Figure 17. T cells expressing KLRG1 in INRs, IRs and HCs. *PBMCs were stained with anti-KLRG1 in order to distinguish the KLRG1+ T cells. Gates were set based on the FMO control for KLRG1. A) Representative flow cytometry images of CD4+ T cells (upper panel) and CD8+ T cells (lower panel) from an INR patient (left) and an IR patient (right). B) Comparison of the percentages of KLRG1+ CD4+ T cells for the three groups. C) Comparison of KLRG1+ CD8+ T cells from the three groups. Lines indicate the mean ± SD.*

4.1.7 INRs show more gut-homing CD4+ T cells than HCs

HIV is a chronic inflammatory disease associated with the gut and studying gut-homing T cells is therefore of great interest. Staining T cells with anti-ITGβ7 allows identification of T cells that home to the gut. The percentages of ITGβ7+ T cells were similar between the groups (SFig. 5). Naive T cells (CD45RA+ ITGβ7+) have the possibility to home to most places in the body [152]. Memory T cells (CD45RA-) that express high levels of the gut homing receptor (ITGβ7 high) are most likely only homing to the gut and these T cells are interesting to study further.

In INR patients, the fraction of memory CD4+ T cells with high expression of ITGβ7 was increased compared to HC (P = 0.0012), but not to the IR (Fig. 18 B upper panel). CD8+ T cells did not show any differences between the groups (Fig. 18B lower panel). Percentages of memory T cells that did not express ITGβ7 were similar between the groups (SFig. 6). Similar percentages of naive ITGβ7+ CD4+ and CD8+ T cells were seen between the groups (Fig. 18C).

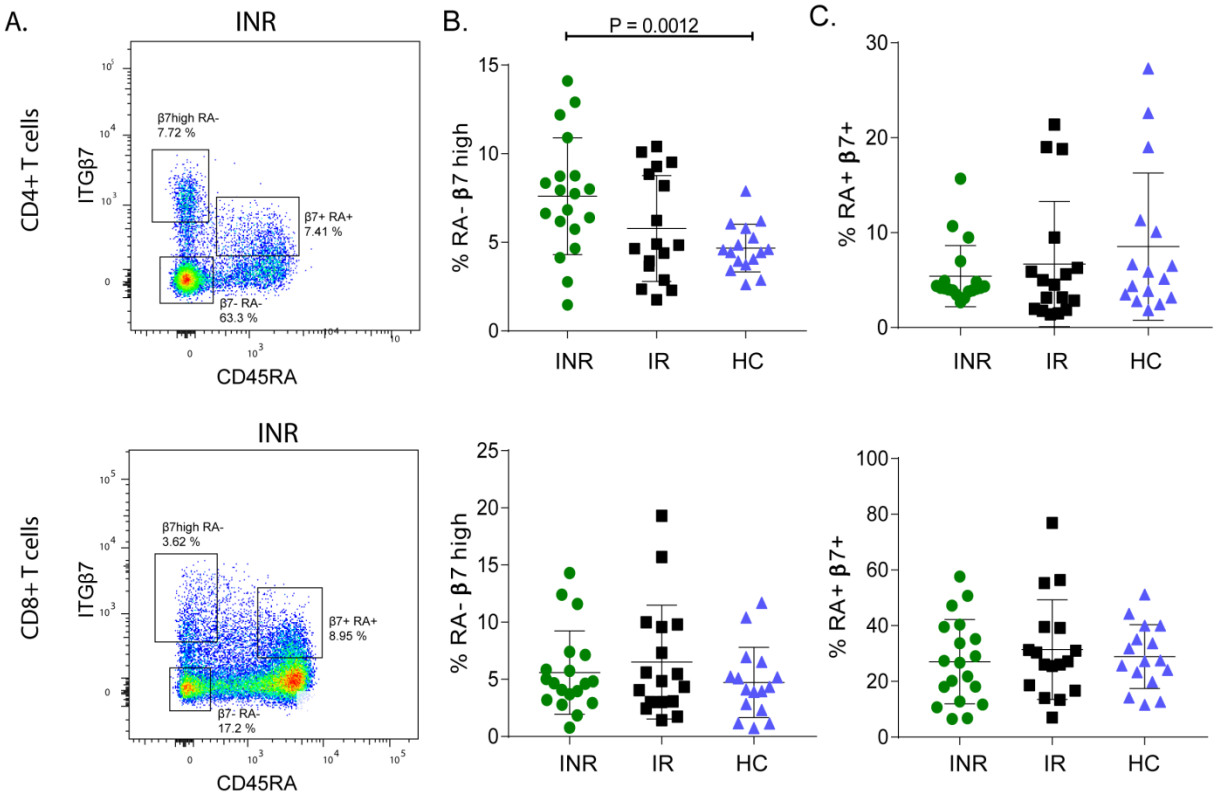


Figure 18. ITGb7 expression on T cells. *PBMCs were stained using anti-ITGβ7 Ab as a surrogate for ITGα4β7 cells. A) Representative flow cytometry images of CD4+ T cells (upper panel) and CD8+ T cells (lower panel). B) Percentages of gut-homing (CD45RA- ITGβ7 high) CD4+ T cells (upper panel), and CD8+ T cells (lower panel). C) Naive (CD45RA+ ITGβ7+) CD4+ T cells (upper panel) and CD8+ T cells (lower panel). P values were calculated with non-parametric Mann-Whitney U-test. Lines indicate the mean ± SD.*

4.1.8 Expression of PD-1 and TIGIT on T cell subsets related to GALT homeostasis

It is also interesting to investigate whether gut homing T cells display a more exhausted phenotype, particularly in INR patients. In order to study that, exhaustion marker expression in the T cell subgroups (naive CD45RA+ ITGβ7+, activated/memory CD45RA- ITGβ7- and gut-homing CD45RA- ITGβ7high) were compared both internally in each group, as well as between the three patient groups. In general, the amount of exhaustion marker expressing cells was lowest in the naive T cells (Fig. 19). With activation, both PD-1+ and TIGIT+ CD4+ T cells were found to be increased regardless of gut homing or not (Fig. 19A and B). For the CD8+ T cells, however, gut-homing T cells seemed to express more PD-1 and TIGIT (Fig. 19C and D). Focusing on the different patient groups, the INR patients showed higher levels of PD-1+ RA- β7- CD4+ T cells than the IR ($P = 0.0014$) and HC ($P = 0.0281$) (Fig. 19A). The fractions of the other CD4+ T cell subsets were similar between the groups (19A). For the CD8+ T cell subsets, INR patients displayed higher fractions of PD-1+ RA- β7high CD8+ T cells compared to IR ($P = 0.0306$) the HC group ($P = 0.0097$). The fractions of the other T cell subsets were similar between the groups (Fig. 19C).

The two memory (CD45RA-) CD4+ T cell subsets both homing to the gut (ITGβ7hi) and not (ITGβ7-) also expressed higher percentages of TIGIT in the INR group compared to HC ($P < 0.0001$) (Fig. 19B). The same T cell subsets were also seen with elevated expression of TIGIT in IR patients compared to HC (Fig. 19B). Still, there were no differences between the INR and IR groups. Regarding CD8+ T cells in INR patients, there were elevated percentages of TIGIT expressing naive RA+ ITGβ7+ ($P = 0.0419$), activated RA- ITGβ7- ($P = 0.0269$) and gut-homing RA- ITGβ7high ($P = 0.0029$) CD8s compared to HC (Fig. 19D). Between INR and IR patients, similar fractions of TIGIT+ CD8+ T cells were seen for all the T cell subsets (Fig. 19D).

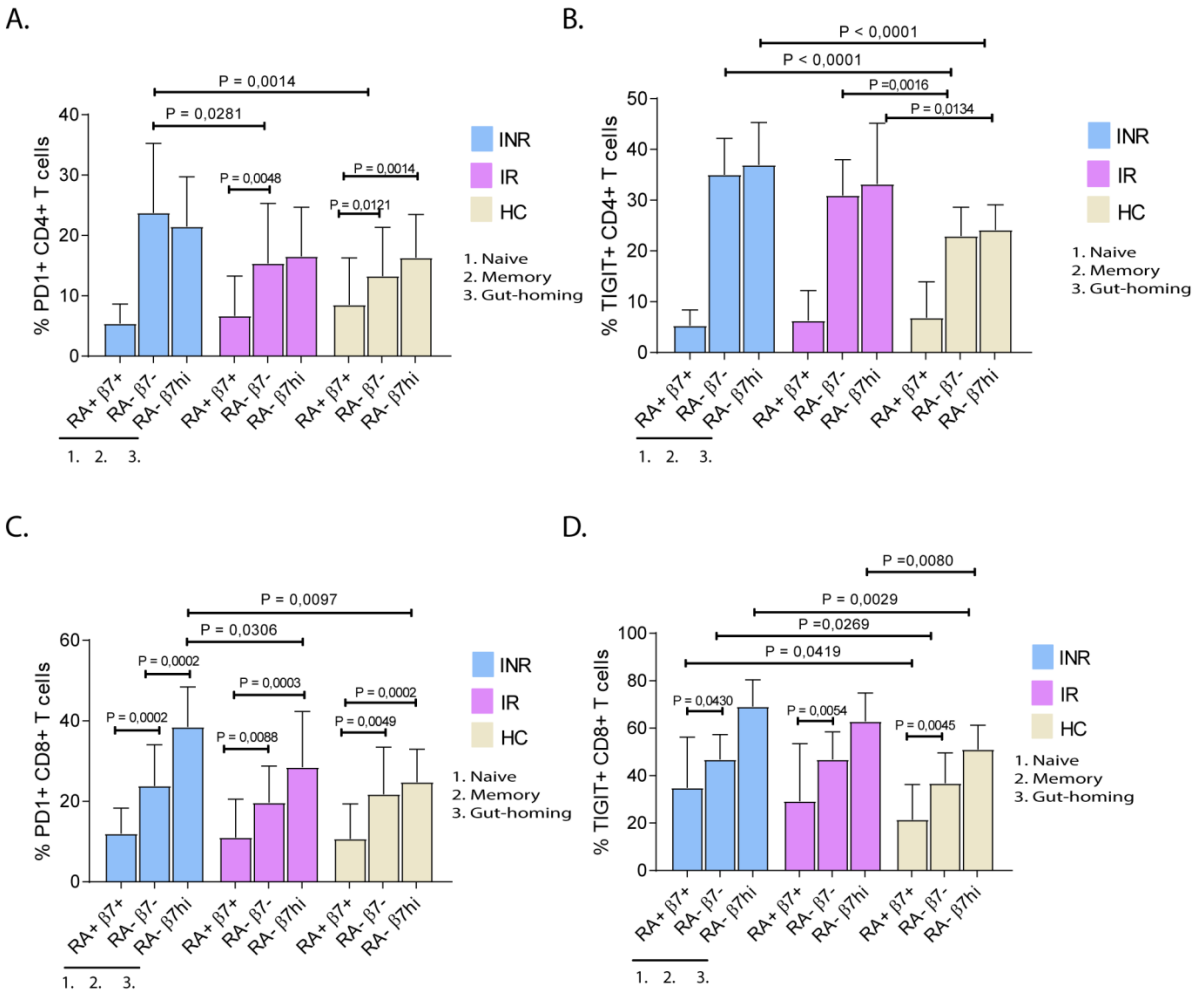


Figure 19. Expression of PD-1 and TIGIT in relation to gut-homing T cells. *In blue are percentages from INR patients, in purple IR patients and in beige the HC group. Comparison of the percentages of (1) naive (CD45RA+ ITGβ7+), (2) memory (CD45RA- ITGβ7-) and (3) gut-homing (CD45RA- ITGβ7high) CD4+ T cells expressing PD-1 (A), and TIGIT (B). Comparison of the percentages of (1) naive, (2) memory, and (3) gut-homing CD8+ T cells expressing PD-1 (C), and TIGIT (D). P values were calculated with non-parametric Mann-Whitney U- test. Lines indicate the mean ± SD.*

The T cell subsets expression of CD57 and KLRG1 were also analysed (SFig. 7). The T cell subsets displayed similar expression of CD57 both internally in each group and between the groups. Expression of CD57 was slightly higher on activated T cells not homing to the gut (CD45RA- ITGβ7-), but this was not a significant increase. Expression of KLRG1 on CD8+ T cells were also similarly expressed both internally and between the groups, except for the HC group were decreased expression of KLRG1 was seen in the naive (CD45RA+ ITGβ7+) T cell group. In contrast to the result with other exhaustion markers, the HC group showed more expression of KLRG1+ naive (CD45RA+ ITGβ7+) T cells compared to INRs (P= 0.0219) and KLRG1+ CD45RA- ITGβ7high compared to INRs (P = 0.0294). Internally in the

INR group, the two activated (CD45RA-) CD4+ T cells subsets homing to gut (ITGβ7high) and not (ITGβ7-) showed higher expression of KLRG1 compared to naive (CD45RA+ β7+) CD4+ T cells.

4.2 Development of a functional assay

4.2.1 Coupling of Dynabeads – finding the optimal concentration of stimulatory and inhibitory signals

Since CD4+ and CD8+ T cells in INR patients had more upregulation of the exhaustion markers PD-1 and TIGIT, we wanted to try to block one or both of these co-inhibitory molecules to see if this could boost T cell function. Several experiments were performed in order to decide which assay conditions that best displayed the effect of PD-1 interactions with PD-L1. To find the optimal combination of co-stimulatory and co-inhibitory signals, anti-CD3/CD28 and PD-L1 were coupled to the beads in two different concentrations. The effect was determined by analyzing cytokine production. To test if the M-450 Epoxy Dynabeads worked properly, PBMCs were for some initial experiments also stimulated with Miltenyi beads coupled with anti-CD3/CD2/CD28, and with PMA + ionomycin as positives controls (SFig. 8).

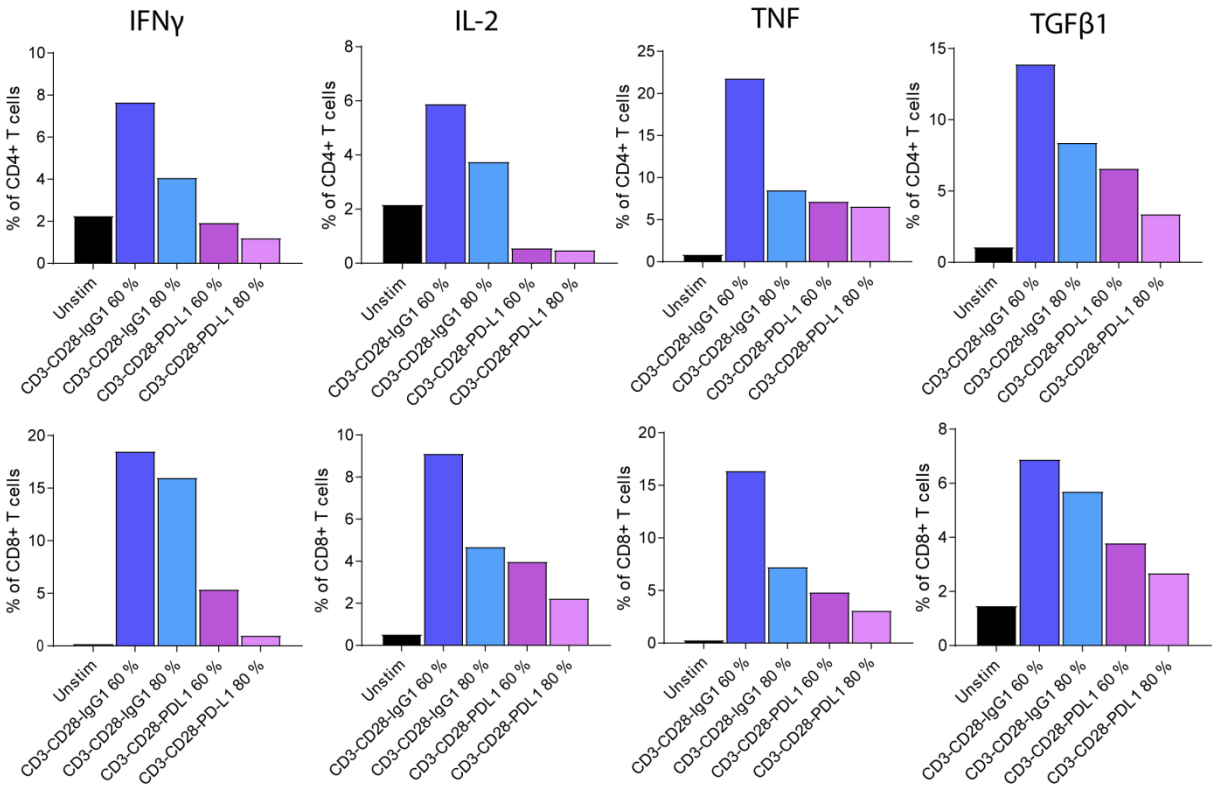


Figure 20. Optimal stimulation/inhibition with beads coupled with 20 % CD3, and 20 % CD28 and 60 % IgG1 or PD-L1. *PBMCs were either stimulated with beads coupled with anti-CD3/CD28 or anti-CD3/CD28/PD-L1 in the presence of Brefeldin A for 6 hours. The cells were fixed and permeabilized before staining with anti-IFNγ, IL-2, TGFβ1 and TNF Abs. We also stained for anti-PD-1 to check the percentages of PD-1+ T cells, as this was important for the results of the stimulation assay (not shown). Unstimulated cells are showed in black, cells stimulated with anti-CD3/CD28 are showed in blue and cells stimulated with anti-CD3/CD28-PD-L1 are showed in purple. The upper part of the figure shows cytokine production in CD4+ T cells, and the lower part shows cytokine production in CD8+ T cells. The data presented is from one representative experiment where HIV+ PBMCs were stimulated.*

Not surprisingly, beads coupled with the highest concentration of co-stimulatory molecules in combination with the isotype control (20 % anti-CD3, 20 % anti-CD28 and 60 % IgG1) gave the highest production of cytokines (Fig. 20). Beads coupled with anti-CD3/CD28 in combination with either 60 or 80 % PD-L1 both inhibited cytokine production (Fig. 20). It was decided to continue with beads coupled with 20 % CD3, 20 % CD28 and 60 % IgG1 or PD-L1, as these concentrations gave the highest cytokine production, and when PD-L1 was added a robust inhibition of cytokine production.

4.2.2 Six-hour stimulation gave the highest cytokine production as well as significant inhibition

Next, the stimulation time was investigated. Activated T cells can produce large amounts of cytokines, but the production can vary depending on the stimulation time. To analyse the production of cytokines, 6 hours and 20 hours of stimulation were tested. The experiment was tested on PBMCs from both healthy blood donors and HIV+ patients (SFig. 9). Six-hour stimulation resulted in more production of cytokines compared to 20 hours stimulation. However, for CD4+ T cell production of cytokines, the differences were only varying with 1-2 percent (Fig. 21, upper panel). Production of cytokines in CD8+ T cells was much higher for INFγ, IL-2 and TNF after 6 hours compared to 20 hours. Stimulation of T cells with PD-L1 also resulted in highest cytokine production after 6 hours but it was only a small difference compared to 20 hours (Fig. 21). Considering all these results together, it was concluded to continue with six-hour stimulation as this, in addition to improving the practical feasibility of the assay, gave the highest cytokine production as well as significant inhibition.

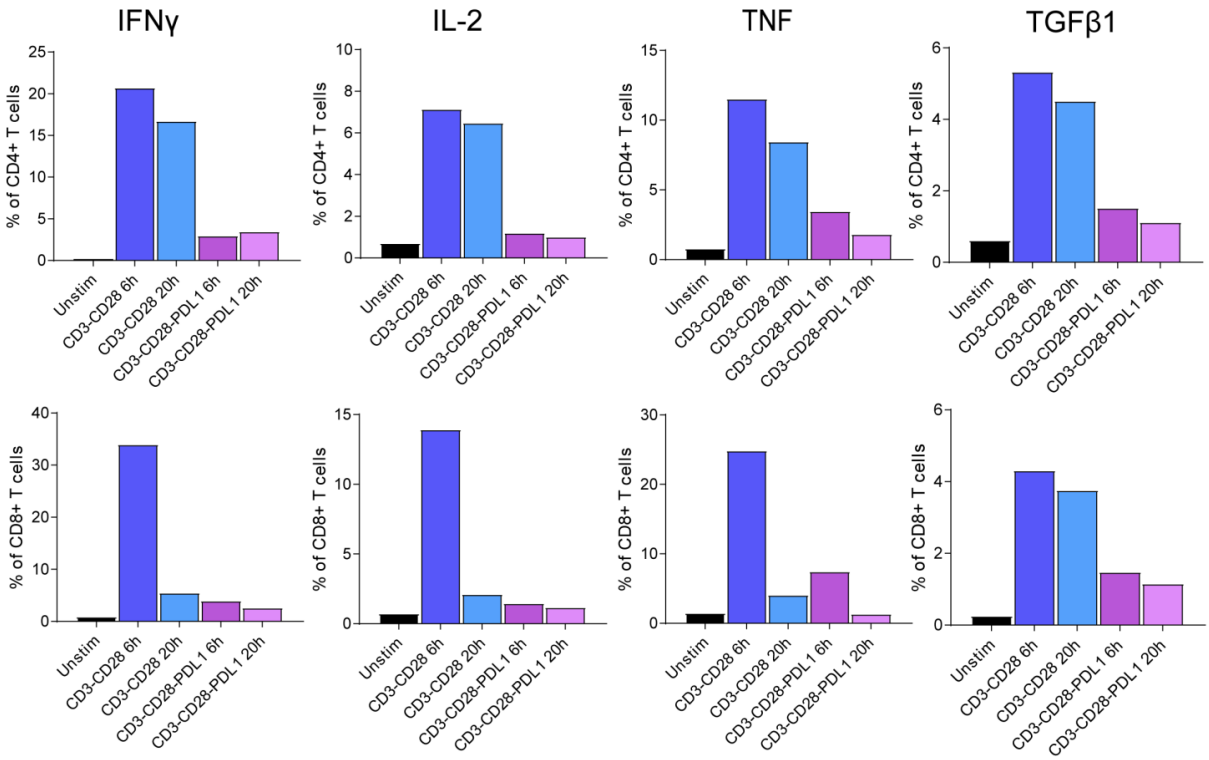


Figure 21. Six-hour stimulation gave the highest cytokine production and significant inhibition. *PBMCs were stimulated for 6 or 20 hours with beads either coupled with ant-CD3/CD28 or anti-CD3/CD28/PD-L1. Brefeldin A was added for 4-6 hours depending on the stimulation time. After 6 and 20 hours the cells were fixed and permeabilized. The next day the cells were stained at the same time with the appropriate Abs. Unstimulated cells are shown in black, cells stimulated with anti-CD3/CD28 in blue and cells stimulated with anti-CD3/CD28/PD-L1 in purple. The upper part of the figure shows CD4+ T cell cytokine production, and the lower part shows CD8+ T cells cytokine production. The data presented is from one representative experiment where HIV+ PBMCs were stimulated.*

4.2.3 Titration of the bead-to-cell ratio

The amount of beads versus cells was next titrated, as an optimal ratio is important in order to activate the T cells but also to be able to see an effect of the inhibitory signal from PD-L1. A bead-to-cell ratio of 10:1 gave the highest production of cytokines for both CD4+ and CD8+ T cells when stimulated with beads only giving co-stimulatory signals. Also ratios of 6:1 and 3:1 resulted in high production of cytokines. Assays with lower bead-to-cell ratios (1:1 and 1:3) did for most cytokines induce production. However, TGFβ1 production was not increased with such ratios (Fig. 22). For stimulations with PD-L1 coupled beads, all ratios resulted in inhibition of cytokine production (Fig. 22). The following experiments were conducted with a bead-to-cell ratio of 3:1.

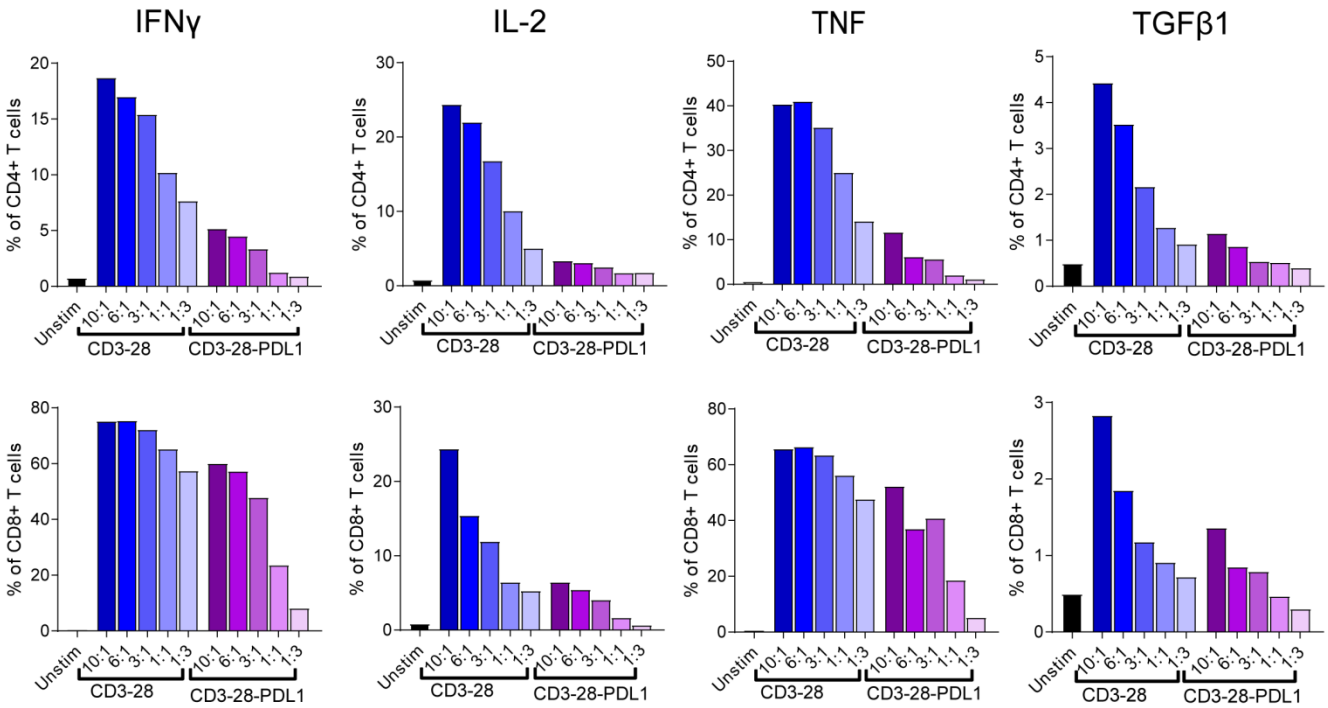


Figure 22. Titration of the bead-to-cell ratio. *PBMCs were stimulated with beads coupled with either anti-CD3/CD28/IgG1 or anti-CD3/CD28/PD-L1 in the presence of Brefeldin A. After 6 hours the cells were fixed and permeabilized, stained and analysed on the flow cytometer. Unstimulated cells are shown in black, cells stimulated with anti-CD3/CD28/IgG1 in different ratios are shown in different shades of blue, and cells stimulated with anti-CD3/CD28/PD-L1 in different shades of purple. The upper part of the figure shows CD4+ T cell cytokine production, and the lower part shows CD8+ T cell cytokine production. The data presented is from one representative experiment where HIV+ PBMCs were stimulated.*

4.2.4 Disruption of the PD-1-PD-L1 interaction with a monoclonal blocking Ab

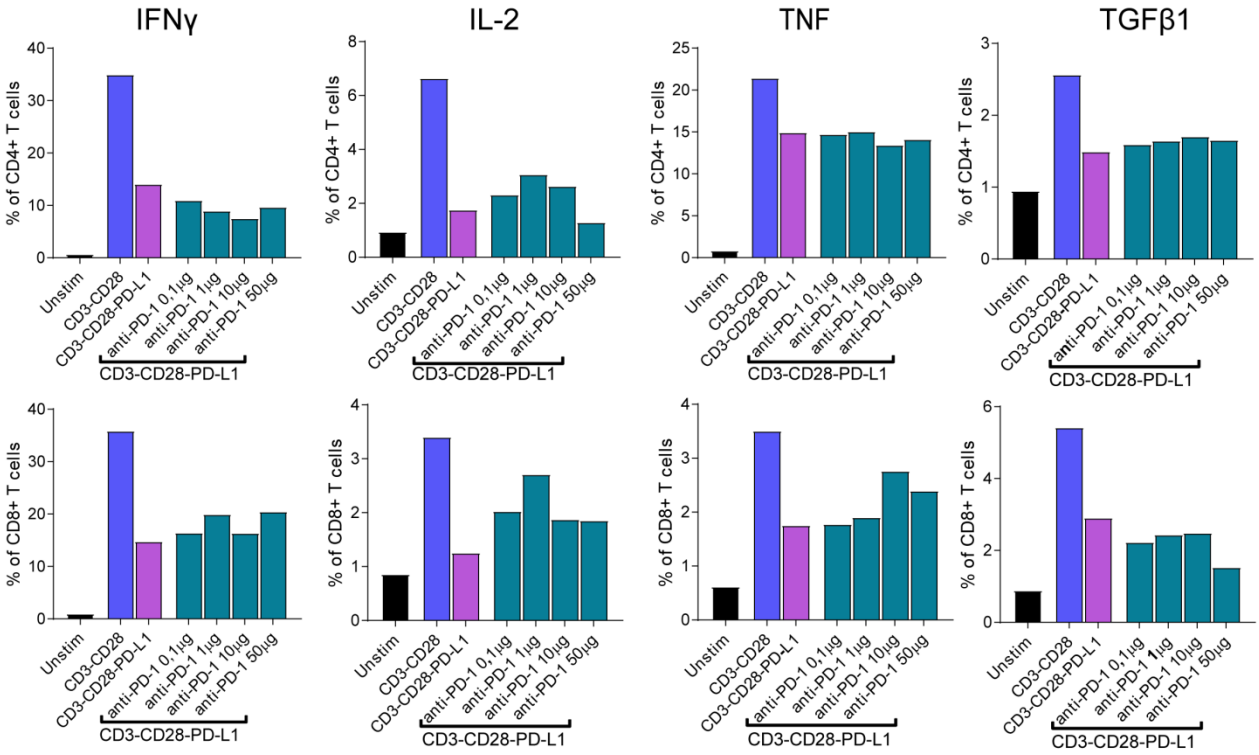


Figure 23. Little or no effect with anti-PD-1. *PBMCs were preincubated with anti-PD-1 one hour before adding the beads to the cell cultures. The cells were then stimulated for 6 hours in the presence of Brefeldin A, before fixation and permeabilization. The cells were stained with appropriate Abs and analysed on the flow cytometer. Unstimulated cells are shown in black, cells stimulated with anti-CD3/CD28 are shown in blue, cells stimulated with anti-CD3/CD28/PD-L1 are shown in purple. The cells incubated with anti-PD-1 and stimulated with anti-CD3/CD28/PD-L1 are shown in turquoise. The upper part of the figure shows CD4+ T cells cytokine production, and the lower part CD8+ T cells cytokine production. The data presented is from one representative experiment where HIV+ PBMCs were stimulated.*

In order to neutralize the inhibitory influence of PD-1, a monoclonal blocking Ab against PD-1 was utilized. Before stimulation with beads, the cells were preincubated with this blocking Ab for 1 hour. Surprisingly, the treatment with anti-PD-1 blocking Ab resulted in little or no upregulation of cytokine production (Fig. 23). There was a slight increase in IL-2 production for both CD4+ and CD8+ T cells, in addition to IFNγ and TNF only for the CD8+ T cells (Fig. 23). However, the cytokine production did not increase in a concentration-dependent fashion with amount of the blocking Ab. No concentrations of blocking Ab was able to

restore cytokine production to the same levels as in the sample stimulated with anti-CD3/CD28/IgG1 beads.

5 Discussion

In this study, a flow cytometry approach was used to compare T cell surface markers in INR patients with IR patients and HCs to determine if INRs display a more exhausted T cell phenotype. The pathogenic basis for INR development is not entirely understood, and therapeutic options to boost immune reconstruction in INR are lacking. A functional assay was established in order to be able to measure the effects of blocking inhibitory pathways for later investigations, to see if this can have an effect on immune reconstruction.

5.1 T cell state

Failure of recovery of CD4+ T cell numbers on ART determines INR status. As expected, INR patients had lower CD4+ T cell counts, and lower ratios of CD4 to CD8 T cells compared to IR patients and HC, which is in line with previous reports [153, 154]. Also, IR showed lower CD4+ T cell counts, and lower CD4/CD8 ratios compared to HC. Previous studies have reported that many effectively treated IR patients achieve sufficient levels of CD4+ T cell counts but still do not reach normal CD4+ T cells levels [155, 156]. In this study, CD8 was not stained for because of limitations in the available detectors on the flow cytometer, as well as trying to minimize spillover of the fluorochrome conjugated to the anti-CD8 Ab into other detectors. Hence, CD8+ T cells were identified as CD3+ CD4-, as well as having an appropriate lymphocyte gate in the forward versus side scatter plot. Thereby we cannot say for certain that the CD8 population gated for in this study is only consisting of CD8+ T cells. Staining with CD3 should exclude B cells and NK cells from the population, but natural killer T cells (NKT) can be a part of this CD4- population. NKT cells express CD3, but also a variety of other markers associated with NK cells such as CD16 and CD56, and these markers have to be stained for in order to exclude them from the CD4- population. There is also a possibility that monoclonal antibodies (mAbs) bind unspecifically to Fc receptors. T cells do not express Fc-receptors, but B cells and monocytes do and since PBMCs were used in our study, FC block should have been used in order to exclude B cells completely. PBS with 2 % FCS was used in this study which will inhibit some of the nonspecific binding.

Several factors have been discussed that may play a role in the pathogenic basis of INR development, such as older age, nadir CD4+ T cell count (the lowest point which the CD4 count has dropped), treatment interruptions and co-infections [119, 123]. In terms of nadir

CD4, the groups of HIV participants in this study, were matched on nadir CD4+ T cell count. Such matching was deliberately chosen in order to investigate if other factors than nadir CD4 could be involved in the process of becoming an INR. This is a strength of this study, as most reports compare INRs and IRs with different nadir CD4.

Previous studies have also shown that INR patients have more T cell activation and effector cells than IR [157, 158], which may lead to increased T cell turnover in patients with low CD4 count. Based on the study by Stiksrud, Lorvik *et al*, it was expected that also for our study INR patients would display less naive T cells and more EM T cells. Surprisingly, this was not observed in our study. INR patients had similar percentages of naive (CD45RA+ CD27+), EM (CD45RA- CD27-), CM (CD45RA- CD27+), and TemRA (CD45RA+ CD27-) T cells compared with IR and HC. A tendency of higher percentage of CD4+ EM T cells are displayed in the INR group, but it was not a significant increase. Also, there were no differences of activated (HLA-DR+ CD38+) T cells between the INRs and IRs. However, the INR group showed a significantly increased percentage of activated CD8+ T cells compared to the HC group. The inconsistency between the study performed by Stiksrud, Lorvik *et al* [157] and our study may be due to the different cohorts of HIV+ participants used in these two studies. Compared to untreated HIV+ patients, HIV patients who receive ART over an extended period of time have shown significantly lower proportions of activated T cells comparable with HIV negative individuals [159]. The patients participating in this study had a median length of treatment of 9.9 years for INR patients and 16.7 for IR patients, which is a higher average than what the cohorts in Stiksrud, Lorvik *et al* had.

Upregulation of co-inhibitory molecules on the cell surface could be a sign of cell exhaustion. However, co-inhibitory molecules are also upregulated when T cells are activated by a pathogen. If the immune system successfully clears the antigen, the co-inhibitory molecules dampen the immune response and the levels of co-inhibitory molecules decrease. When the immune system is not able to clear the infection, which is seen in chronic infections and some types of cancers, expression of co-inhibitory molecules remains high and sustained and eventually leads to T cell exhaustion. The percentage of T cells that expressed PD-1 was significantly increased in INR patients compared with IR and HC. INR patients participating in this study successfully suppressed the virus, indicating that the INRs have persistent elevated PD-1 expression despite effective ART. Elevated expression of PD-1 on T cells can lead to T cell exhaustion and may result in increased apoptosis and intrinsic T cell death

[160]. Previously reports have shown that suppression of viral loads in patients who are receiving ART, usually also result in downregulation of PD-1 on T cells [161]. These results are in line with the results in this study which show similar percentage of PD-1+ T cells between IR and HC.

TIGIT+ T cells were also significantly increased in INRs compared with IRs and HCs. These findings support that INR patients display a more exhausted phenotype compared to IRs. However, IRs also had an increased percentage of TIGIT+ CD4+ T cells compared to HCs, suggesting that also IRs have a more exhausted T cell phenotype than HIV negative individuals. Previous reports by Chew *et al* and Tauriainen *et al* showed that expression of TIGIT is increased over time despite early initiation of ART, suggesting that a decrease in viral load alone does not fully restore the T cells [139, 162]. Still, the results of this study indicate that INR patients display a more severely exhausted T cell phenotype compared to IRs, suggesting that also TIGIT, in addition to PD-1, can be related to insufficient CD4 reconstitution.

Interestingly, INRs also showed significantly increased percentages of T cells that dually expressed PD-1 and TIGIT when compared with IRs and HCs. A significantly higher percentage of PD-1+ TIGIT+ CD4+ T cells was also observed for IR patients compared with the HC group and a tendency to the same was seen for the CD8+ T cells. These results strengthen our findings that INR patients display a more exhausted T cell phenotype compared to IRs, but also that HIV patients in general express more PD-1 and TIGIT compared with uninfected controls. The negative correlation we found between PD-1+ TIGIT+ T cells was not seen when we analysed the three patients groups independently. There could be several reasons for this. One reason could be that there was a low number of samples in each group. Another reason could be due to group effects or class effects (CD4 numbers are not a linear variable between the groups). In addition to studying the percentages of exhaustion marker-expressing cells, it is also interesting to analyse if cells also have increased expression of such markers. That can be done by looking at fluorescence intensity. A comparison of geometric fluorescence intensity analysis was performed, but did not show any differences in expression of either activation or exhaustion markers. Hence, even though INR patients display more T cells expressing e.g. PD-1, these cells did not have higher amounts of PD-1 on the surface. Still, as INRs had relatively more T cells expressing exhaustion markers, these results suggest that PD-1 and TIGIT may play a role in the

insufficient immune reconstitution. However, this does not answer whether PD-1 is (a part of) the primary reason for the immunologic failure or if it is a consequence of the disease.

Surprisingly, no differences were seen between the INR, IR, and HC groups in T cells expressing Lag-3, Tim-3 and CD160. All groups displayed low expression of these co-inhibitory molecules. These results may be due to biological aspects or because of technical difficulties. Lag-3, Tim-3, and CD160 have been demonstrated to be upregulated in dysfunctional and exhausted T cells in HIV patients, but previous studies have also reported that co-inhibitory molecules often are downregulated again after long-term ART. Lag-3 expression was reported by Tian *et al* to be reduced on both CD4+ and CD8+ T cells in HIV patients with prolonged ART [163] but Lag-3 has also been reported to be significantly expressed together with PD-1 and TIGIT in HIV patients receiving ART [164] so it is difficult to determine whether different results are caused by different cohorts of patients or technical errors. Still, we did expect to find higher levels of several exhaustion markers in INRs as this patient group is believed to have a more pronounced exhaustion phenotype than successfully treated HIV patients. Expression of Tim-3 and CD160 has been reported to be downregulated in HIV patients on ART [164, 165]. However, for Tim-3, studies have also reported that Tim-3 can be seen highly expressed in long-term treated patients, but this was associated with high levels of T cells activation (CD38+ HLA-DR+) [165]. In our study patient cohort, higher levels of CD38+ HLA-DR+ cells were not seen in the HIV patients, suggesting that Tim-3 may be downregulated in this patient cohort.

It cannot be ruled out that the low expression of Lag-3, Tim-3 and CD160 observed in this study may be due to technical difficulties. Even though two different clones were tested for each antibody, several factors concerning the optimization of the Ab staining panel may have caused problems such as unspecific binding of Abs to other epitopes. To get an accurate measurement of fluorescence, it is important to maximize the signal while reducing the noise. Titrations of Abs were done in order to find the optimal concentration that would give a bright signal with lowest possible background signal. An Ab will bind with high affinity to epitopes on the specific target. If too high amount of one Ab is used, more Ab than high-affinity epitopes will be present, and the Ab will start to bind low-affinity targets [166]. This is more often a problem seen for intracellular staining, but it can also occur on the cell surface. Another reason can be fluorescence spillover. This can often be a problem in multicolor experiments. Fluorescence spillover occurs when light emitted from one fluorochrome is

registered in multiple detectors which can lead to a reduction of sensitivity (false positive signals) [167]. We had up to 15 colors in our panel. The panel was thoroughly planned with regards to low-expressing markers being stained with brighter fluorochromes and vice versa, as well as having the appropriate filters in the flow cytometer. Despite this, we cannot rule out the possibilities that the staining for some markers was not optimal.

Long-term treated HIV+ persons usually die at an earlier age than uninfected persons and suffer from non-AIDS diseases usually seen later in life. Previous studies have suggested that HIV infected persons suffer from premature aging leading to increased susceptibility of infections, decreased protection from previous and new vaccinations and increased risk of malignant diseases in several organs such as the heart, liver and kidney. INR patients have an even higher risk of developing non-AIDS diseases and low CD4+ T cell counts can be the reason for this [168]. Studying immunosenecence markers such as CD57 and KLRG1 can yield insight into the role of immunosenecence in HIV+ patients. In this study, it was investigated whether INR patients expressed increased levels of CD57 and KLRG1 compared to IRs. The analysis showed similar expression of CD57 and KLRG1 in the three groups, which indicates that INRs do not display a more senescent T cell phenotype compared to IR patients and uninfected controls. Previous studies with elderly persons and CMV infection have demonstrated that this group has increased levels of CD8+ T cells with downregulated CD28 and upregulated CD57. Lee *et al* reported that HIV+ patients also showed increased expression of CD28- CD8+ T cells, but these cells did not increase expression of CD57 [169]. Tavenier *et al* reported that KLRG1 expression did not differ between HIV+ and uninfected controls and that immunosenecence seemed to be highly dependent on HIV-infection and is only to a small extent associated with age-related parameters in well-treated HIV infections [170]. None of these studies compared INR patients with IR, but since our study showed no differences between the two HIV+ patient groups, this indicates that immunosenecence does not play a role in the insufficient reconstitution of CD4+ T cells in INRs. Notably, CD28 was not stained for in this study, which is limiting this study's possibility to fully investigate immunosenescence, as CD28 in combination with CD57 would give a more accurate characterization of the T cells. It would have been interesting to see if INR patients have a higher percentage of CD28- CD57+ T cells compared to IR.

5.2 HIV is a gut associated disease

The HIV envelope can bind to the ITGα4β7 receptor via gp120, which enables HIV to colonize the GALT, for further development of an intestinal HIV reservoir. CD4+ T cells expressing ITGα4β7 are highly depleted in early stages of infection and also during infection. ITGβ7+ (used as a surrogate for ITGα4β7) T cells have been studied in this Thesis to see if there is any correlation between the gut homing receptor and low CD4+ T cell reconstitution in INRs. Immune cells can home to effector sites in the GALT by expressing ITGα4β7 which will interact with its ligand MADCAM1. MADCAM1 is expressed on high endothelial venules in the gastrointestinal tract. ITGβ7 can also form a dimer with ITGαE. Intraepithelial lymphocytes express ITGαEβ7 and by interaction with epithelial cells, ITGαEβ7 lymphocytes can home to the skin. In our study ITGα4 was not stained for and therefore we cannot say for certain that our ITGβ7 population only includes cells homing to the gut. Previous studies have demonstrated that T cells being ITGβ7high are > 99 % ITGα4β7+ [171-173]. Also preliminary analyses done before our main study, where HIV+ PBMCs were stained with both ITGαE and ITGβ7 showed that less than 3 % of the T cells were double positive for ITGαE and ITGβ7 indicating that most T cells expressing ITGβ7 forms a dimer with ITGα4.

Our study showed similar expression of ITGβ7+ CD4+ and CD8+ T cells between the groups, but INRs showed a significantly increased percentage of CD45RA- ITGβ7high CD4+ T cells compared to HCs, and a tendency of increased percentages compared to IRs. These results indicate that INRs display more gut-homing CD4+ T cells. However, we could not detect any differences in percentages of CD45RA- ITGβ7high CD8+ T cells. We can speculate if INR patients have a more severe gut-associated inflammation with increased leaky gut and further recruitment and depletion of CD4+ T cells. Also, a possible higher recruitment of T cells to the gut may not be detected in our study as we were actually looking at blood and not gut tissue. If the cells have already migrated into the tissue, this might not be reflected in the blood. During recruitment of patients to this study, biopsies from the colon were sampled in addition to blood, and it will be very interesting to study the gut-associated T cells in future studies.

In contrast to our study, Girard *et al* reported that INRs expressed more of both CD4+ and CD8+ T cells expressing ITGβ7 alone as well as CD45RA- ITGβ7+ CD4+/CD8+ T cells compared to IR and HC [174]. The inconsistency may be due to differences in the cohorts of patients. Girard *et al* define the cohorts by undetectable plasma viral load < 40 copies/mL for

an average of 2,8-6,9 years for INR patients and 4,1-6,5 years for IR patients [174]. The article does not define the average length of treatment, but defines the average length of viral load < 40 copies/mL. Based on the number from the viral suppression it seems like the average length of treatment is shorter for the patients in the study of Girard *et al* compared to our study. This suggests that the patient groups in our study had decreased activation and increased restored gut CD4+ T cells for both INRs and IRs due to longer effective ART compared to the cohort of patients used in Girard *et al* study. Another study by Ciccone *et al* demonstrated that prolonged ART (cohort of HIV participants with a median length of 8 years of plasma viral load < 50 copies/mL) restores the gut CD4+ T cell population and that this is reflected in ITGβ7 expression on circulating CD4+ T cells [175]. The study by Ciccone *et al* compared IR patients to untreated patients so it cannot be fully compared to this study, but these reports suggest that reconstitution of gut CD4+ T cells correlates with length of treatment for both INR and IR patients and may not contribute to the insufficient level of CD4+ T cell count in long-term treated INR patients. However, a lack of long-term prospective studies in this field makes it difficult to conclude. Our study and the other reports by Girard *et al* and Ciccone *et al* has solely examined the T cells in the circulating system, and since the determining events of HIV infections mostly take place in the GALT, more research from gut-homing T cells is necessary to conclude whether INR patients show a more exhausted T cell phenotype in gut-associated T cells. Since INRs show increased levels of activated CD4+ T cells homing to the gut compared to HCs, it was interesting to analyse if INR patients showed increased expression of exhaustion markers such as PD-1, TIGIT, CD57 and KLRG1 in the same T cell subsets. Naive T cells expressing intermediate levels of ITGβ7 (CD45RA+ ITGβ7+) had the lowest percentages of T cells expressing PD-1 and TIGIT in all three patient groups. Naive T cells showed significant decrease of T cells expressing PD-1 and TIGIT compared to the two activated T cell subsets. Comparing the study groups, naive T cells expressed similar percentages of PD-1+ T cells and TIGIT+ T cells. These results indicate that INR patients do not display a more exhausted naive T cell population compared to IRs and HCs.

When it comes to the activated/memory cells homing or not to the gut, the CD4+ T cells did not display any differences in exhaustion marker expression internally in the three patient groups. Interestingly, the INR patients had slightly higher PD-1 and TIGIT expression of the CD45RA- ITGβ- CD4+ T cells than the two other groups, indicating that there are more systemic inflammation in INR patients affecting a greater proportion of the general CD4+ T

cells pool (and not only T cells homing to the gut). On the other hand, we found that the gut homing CD8+ T cells expressed the most PD-1 and TIGIT, and that the percentages of these cells were highest amongst INRs.

With regards to senescence, CD57 was neither differentially expressed neither between the patient groups nor internally in the individual groups. However, the amount of CD57 high T cells correlated negatively with CD4 percentages. This indicated that even though INR patients did not have more CD57+ T cells, there is a relationship between having low CD4 numbers and senescent cells. Naive and activated T cells expressing ITGβ7 or not showed similar expression of KLRG1 for all the groups both internally and between the groups for CD8+ T cells but interestingly activated CD4+ KLRG1+ T cells homing to the gut were increased in IRs and HCs compared to the INR group that showed the least KLRG1+ T cells for the three T cell subsets. The reason for this is not clear, but our data suggest that KLRG1 expression might correlate with normal immune activation seen in all persons and is not upregulated in response to a leaky gut.

5.3 Functional in vitro T cell stimulation assays

In our study, not only HIV-specific T cells were analysed, but the general pool of T cells. It is interesting to study the whole pool of T cells because even though productive infection in CD4+ T cells leads to apoptotic death, many CD4+ T cells not infected also die by bystander apoptosis [176, 177]. It is yet not clear what causes this bystander killing but the Env glycoprotein from HIV is believed to be implicated. Env proteins which are expressed on infected cells, are able to bind to uninfected T cells expressing CD4 and CXCR4/CCR5. Also mechanisms such as host-derived factors like TNF and Fas-ligand but also viral factors like Tat, Vpr and Nef released from infected cells can cause apoptosis [178]. Our study shows that the general T cell pool of INR patients is exhausted which also causes T cell depletion and that blocking exhaustion markers may increase the CD4 T cell pool.

PD-1 Abs are now being investigated for their ability to reverse immune exhaustion. Blocking the PD-1-PD-L1 pathway is established treatment as immunotherapy in some cancers [179, 180], and several studies suggest that inhibiting PD-1/PD-L1 interactions can lead to increased CD8 T cell proliferation, cytokine production, and cytotoxic activity, all in all to a more efficient viral control [181-183]. Blocking this pathway may also improve CD4+ T cell count by increasing the number of T cell proliferation [178]. Better effector function of HIV-

specific T cells has proven to enhance and restore virus-specific T cell responses. A very important aspect of boosting T cell proliferation in HIV+ patients is that more proliferating T cells can lead to more CD4+ T cells that the virus can infect. Taking this into consideration, immunotherapy for HIV+ patients must most likely be given together with ART in order to continue suppression of viral replication.

The functional *in vitro* T cell stimulation assay established in this study was done to try to block PD-1 and TIGIT something that if effective ultimately could be used to counteract T cell exhaustion and to reconstitute CD4+ T cells numbers in INR patients. PBMCs from both healthy and HIV+ patients were included on order to exclude the pathogenic factors following the HIV infections as the reason for poor cytokine production. M-450 Epoxy Dynabeads coupled with anti-CD3/CD28/IgG1 was used to stimulate T cells (IgG1 was used as a mock for PD-1). Six-hour stimulation gave higher percentages of cells with cytokine production compared to 20 hours. To study the effects of PD-1, anti-CD3/CD28-PD-L1 was coupled to another set of beads. The literature states that a concentration of approximately 10-20 % anti-CD3 and anti-CD28 coupled to beads gives an optimal activation and 60 - 80 % PD-L1 coupled to beads gives an optimal inhibition of T cell activation [179, 184]. Anti-CD3 and anti-CD28 concentrations that were tested in this study was 10 % and 20 % and it was observed that the beads with 60 % IgG1 (20 % anti-CD3, 20 % anti-CD28) gave higher percentages of cytokine production (IFNγ, IL-2, TNF and TGFβ1) compared to 80 % (10 % anti-CD3, 10 % anti-CD28). The experiments executed to test concentration had a stimulation time of 6 hours, which might have affected the results. The beads with only 10 % anti-CD3 and 10 % anti-CD28 (80 % IgG1) might have shown higher cytokine production with longer stimulation time. Ideally, all parameters should have been tested in the same set up. 60 % and 80 % concentration of PD-L1 also showed similar percentages of inhibition for CD4+ T cells, but for CD8+ T cells, beads with 60 % PD-L1 showed slightly better inhibition capacity. When repeating this experiment, similar results was observed and it was concluded to proceed with 60 % concentration of IgG1/PD-L1.

After coupling the Dynabeads, they were tested in an experiment together with cells stimulated with Miltenyi beads and cells stimulated with PMA + ionomycin (as the two latter methods are routinely and successfully used in the lab). Dynabeads use a different coupling method than the biotin-binding Miltenyi beads. The Epoxy Dynabeads bind covalently to amino- and sulphydryl groups in proteins, and it is not certain that all bound proteins will be

coupled to the beads with the epitope binding areas outward from the bead. If poor cytokine production was observed, having some control stimulatory agents could reassure us that it was not the Dynabeads that were not functioning, but rather that the cells were less responsive.

Not surprisingly the cells stimulated with PMA and ionomycin gave the highest production of cytokines, except for CD4+ and CD8+ T cell production of IL-2 and TGFβ1. The stimulation time was 20 hours, and this may have been too long for certain cytokines, as for example IL-2 is produced at the highest amount 2-4 hours after stimulation. T cells stimulated with Dynabeads gave similar production of IFNγ, and TGFβ1 for both CD4+ and CD8+ T cells and increased cytokine production of IL-2 compared with T cells stimulated with Miltenyi beads. These results indicated that the coupling of anti-CD3/CD28 to the Dynabeads was successful.

The literature also states that different beads-to-cell ratios have been used in order to get an optimal stimulation. Bead-to-cell ratio of 10:1, 3:1 and 1:1 have all been observed to give an optimal stimulation and inhibition of T cell activation [182, 184, 185]. Several experiments where bead-to-cell ratios were titrated showed high percentages of cytokine production for 10:1, 6:1 and 3:1 ratios for both CD4+ and CD8+ T cells. Also assays with lower bead to cell ratios (1:1 and 1:3) resulted in cytokine production but at a lower scale (except for TNF) compared to the higher ratios. Stimulation with PD-L1 coupled beads resulted in inhibition of cytokines for all the ratios tested. Considering that previous studies have shown that 3:1 gave a sufficient T cell activation and cytokine production, and having in mind that 10:1 and 6:1 ratios may give such powerful T cell stimulation that it would be difficult to see the effect of the inhibition with PD-L1, the ratio 3:1 was selected to be used for future experiments.

In order to further characterize the contribution of various inhibitory pathways in functional T cells, anti-PD-1 was tested to see if mAbs could block the interaction between PD-1 and PD-L1. Unfortunately, preliminary experiments showed little restoration of cytokine production. Blocking exhaustion markers are frequently done in many research fields, so this is most likely due to a technical issue. Maybe the anti-PD-1 used in these experiments binds to an epitope on the PD-1 receptor that doesn't fully block the PD-L1 to interact to PD-1, suggesting that other clones of anti-PD-1 or anti-PD-L1 mAbs should be tested. Different amounts of anti-PD1 may also be tested in a new titration assay to investigate if this has any effect.

5.4 Limitations with this flow cytometry approach

The flow cytometry approach used in this study comes with limitations. Due to limited patient material and many markers included in the panel, FMOs were combined as fluorescence minus 3 controls. This might not be as ideal as having one FMO control for all Ab. Experiments were done prior to this study to test if the percentage of T cells expressing different cell surface markers varied when markers were controlled for with true FMOs or in combinations. These results (which are not shown in this Thesis) showed that making the controls as fluorescence minus 3 gave slightly lesser background than true FMO controls. Preliminary data showed that the background for certain markers, such as CD38 and HLA-DR, varied from donor to donor. Hence, it was necessary with individual controls for these two markers. However, for some donors we had too few cells to be able to divide the sample into all necessary FMO controls.

Instead, it was decided to use fluorescence minus 3 to have controls for all markers and donors. As all donors had individual fluorescence minus 3 controls and that all samples where gated in the same way, we argue that such a gating/control strategy can be accepted. Indeed, how to set up an experiment with FMO and the necessity of individual controls are debated [186]. There are several factors to take into consideration, such as sample material, work load and costs. We could have included isotype controls into the FMO in order to account for unspecific staining signals from the fluorochrome of interest. However, we did not include this. Flow cytometry is limited by the availability of lasers and detectors, as well as Abs with the appropriate conjugate provided from companies. There are several other markers we could have analysed, but which had to be excluded due to the factors listed above. For future experiments it would be interesting to study other co-inhibitory receptors such as CTLA-4 and BTLA to see if they are more upregulated in INRs compared to IRs. Also including anti-CD8 and anti-CD45RO Abs would have been good to conclude which T cells that truly was CD8+ (not just CD4-) and CD45RO+ (not just CD45RA-).

Another aspect with flow cytometry is the gating process. Even though one can use FMO and other controls, the gating can sometimes be difficult and slightly subjective. Also, for some plots and markers there are no controls, e.g. for the lymphocytes in the forward versus side scatter plot. We had a very strict lymphocyte gate that could have been bigger, especially since we were studying exhausted cells that might have a different cell size than naive and

activated lymphocytes. Ideally, all analyses should have been done with a bigger lymphocytes gate as well, to see if there were any differences in expression of the various surface markers.

All experiments executed in this study were done on frozen PBMCs. Flow cytometry analysis done on cryopreserved PBMC (cPBMC) can affect the results. The cryopreservative the cells are frozen in can result in downregulation of surface and intercellular markers, change the cells behavior and decrease cell viability. Therefore resting PBMCs after thawing is an option before intracellular and cell surface staining [187]. Resting of thawed PBMCs usually increases the function of T cells, makes the cells more robust, increase viability and decrease cytokine background noise. In this study PBMCs were rested overnight (approximately 18 hours) and they usually showed viability over 90 % after rest. However, it is important to be aware that resting might change the phenotype of the cells compared to the *in vivo* situation. Using fresh PBMC would have been the ideal situation. Unfortunately, collecting sample material from many individuals takes time, and if you want to perform the analysis under similar circumstances, the biological material needs to be frozen and stored until the inclusion is finished.

All in all, I would state that flow cytometry is a very good approach in order to answer the questions we had in this study. Flow cytometry gives an advantage over several other methods as we can extract lots of information at a single cell level.

5.5 Conclusion and future perspectives

In summary, we could not observe any difference between the three groups when it comes to activation and differentiation (Aim 1). However, INR patients showed a more exhausted T cell phenotype compared to IRs and HCs by having higher percentages of T cells expressing PD-1 and TIGIT alone, as well as a dual expression of both PD-1 and TIGIT. The expression of several exhausted and senescence markers was negatively correlated with CD4 percentages (Aim 2). The functional assay developed in this study gives us the opportunity to analyse the effect PD-1 ligation has on T cells (Aim 3). However, there is a need for further optimization in order to study the effect of blocking the co-inhibitory molecules.

Future perspectives following on the work of this Thesis are to look at HIV-specific T cells, not just the general T cell population, in order to see if HIV-specific T cells in INR patients also show a more exhausted T cell phenotype compared to IR patients. Upregulation of

exhaustion markers will inhibit signaling cascades from the TCR leading to inhibition of T cell activation. Future perspectives will also involve studying intracellular signaling to see if cells expressing co-inhibitory receptors such as PD-1 and TIGIT have less phosphorylation of molecules such as Akt, Mek, Zap-70, and Lck, and to eventually try to block the engagement of PD-1 and PD-L1 to boost T cell functions.

6 References

1. Parham, P., *The Immune System*. Vol. 4th edition. 2015: Garland Science, Taylor & Francis Group, LLC.
2. Fontenot, A.P. and P.L. Simonian, *13 - Adaptive Immunity A2 - Broaddus, V. Courtney*, in *Murray and Nadel's Textbook of Respiratory Medicine (Sixth Edition)*, R.J. Mason, et al., Editors. 2016, W.B. Saunders: Philadelphia. p. 206-224.e3.
3. Zuniga-Pflucker, J.C., *T-cell development made simple.* Nat Rev Immunol, 2004. **4**(1): p. 67-72.
4. Germain, R.N., *T-cell development and the CD4-CD8 lineage decision.* Nat Rev Immunol, 2002. **2**(5): p. 309-22.
5. Starr, T.K., S.C. Jameson, and K.A. Hogquist, *Positive and negative selection of T cells.* Annu Rev Immunol, 2003. **21**: p. 139-76.
6. Takaba, H. and H. Takayanagi, *The Mechanisms of T Cell Selection in the Thymus.* Trends Immunol, 2017. **38**(11): p. 805-816.
7. Mackay, C.R., *Homing of naive, memory and effector lymphocytes.* Current Opinion in Immunology, 1993. **5**(3): p. 423-427.
8. Luckheeram, R.V., et al., *CD4+T Cells: Differentiation and Functions*. Vol. 2012. 2012. 12.
9. Constant, S.L. and K. Bottomly, *Induction of Th1 and Th2 CD4+ T cell responses: the alternative approaches.* Annu Rev Immunol, 1997. **15**: p. 297-322.
10. Zhu, J., H. Yamane, and W.E. Paul, *Differentiation of effector CD4 T cell populations (*).* Annu Rev Immunol, 2010. **28**: p. 445-89.
11. Wan, Y.Y. and R.A. Flavell, *How diverse--CD4 effector T cells and their functions.* J Mol Cell Biol, 2009. **1**(1): p. 20-36.
12. Hatada, M.H., et al., *Molecular basis for interaction of the protein tyrosine kinase ZAP-70 with the T-cell receptor.* Nature, 1995. **377**(6544): p. 32-8.
13. Cary, L.A. and J.A. Cooper, *Molecular switches in lipid rafts.* Nature, 2000. **404**(6781): p. 945, 947.
14. Deindl, S., et al., *Structural basis for the inhibition of tyrosine kinase activity of ZAP-70.* Cell, 2007. **129**(4): p. 735-46.
15. Cloutier, J.F. and A. Veillette, *Cooperative inhibition of T-cell antigen receptor signaling by a complex between a kinase and a phosphatase.* J Exp Med, 1999. **189**(1): p. 111-21.
16. Skanland, S.S., et al., *T-cell co-stimulation through the CD2 and CD28 co-receptors induces distinct signalling responses.* Biochem J, 2014. **460**(3): p. 399-410.
17. Acuto, O., V. Di Bartolo, and F. Michel, *Tailoring T-cell receptor signals by proximal negative feedback mechanisms.* Nat Rev Immunol, 2008. **8**(9): p. 699-712.
18. Smith-Garvin, J.E., G.A. Koretzky, and M.S. Jordan, *T Cell Activation.* Annual review of immunology, 2009. **27**: p. 591-619.
19. Hutloff, A., et al., *ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28.* Nature, 1999. **397**(6716): p. 263-6.
20. Nunes, J.A., et al., *Signal transduction by CD28 costimulatory receptor on T cells. B7-1 and B7-2 regulation of tyrosine kinase adaptor molecules.* J Biol Chem, 1996. **271**(3): p. 1591-8.
21. Fraser, J.D. and A. Weiss, *Regulation of T-cell lymphokine gene transcription by the accessory molecule CD28.* Mol Cell Biol, 1992. **12**(10): p. 4357-63.
22. Appleman, L.J. and V.A. Boussiotis, *T cell anergy and costimulation.* Immunol Rev, 2003. **192**: p. 161-80.

23. Okin, D. and R. Medzhitov, *Evolution of Inflammatory Diseases.* Current biology : CB, 2012. **22**(17): p. R733-R740.
24. Punchard, N.A., C.J. Whelan, and I. Adcock, *The Journal of Inflammation.* Journal of Inflammation (London, England), 2004. **1**: p. 1-1.
25. Lawrence, T. and D.W. Gilroy, *Chronic inflammation: a failure of resolution?* International Journal of Experimental Pathology, 2007. **88**(2): p. 85-94.
26. Sharp, P.M. and B.H. Hahn, *Origins of HIV and the AIDS Pandemic.* Cold Spring Harbor Perspectives in Medicine:, 2011. **1**(1): p. a006841.
27. Nyamweya, S., et al., *Comparing HIV-1 and HIV-2 infection: Lessons for viral immunopathogenesis.* Rev Med Virol, 2013. **23**(4): p. 221-40.
28. Al-Jabri, A.A., *How does HIV-1 infect a susceptible human cell?: Current thinking.* Journal for scientific research. Medical sciences / Sultan Qaboos University, 2003. **5**(1-2): p. 31-44.
29. Peeters, M., C. Toure-Kane, and J.N. Nkengasong, *Genetic diversity of HIV in Africa: impact on diagnosis, treatment, vaccine development and trials.* Aids, 2003. **17**(18): p. 2547-60.
30. *Kaposi's sarcoma and Pneumocystis pneumonia among homosexual men--New York City and California.* MMWR Morb Mortal Wkly Rep, 1981. **30**(25): p. 305-8.
31. Gottlieb, M.S., et al., *Pneumocystis carinii pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency.* N Engl J Med, 1981. **305**(24): p. 1425-31.
32. Barre-Sinoussi, F., et al., *Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS).* Science, 1983. **220**(4599): p. 868-71.
33. Hammer, S.M., et al., *A controlled trial of two nucleoside analogues plus indinavir in persons with human immunodeficiency virus infection and CD4 cell counts of 200 per cubic millimeter or less. AIDS Clinical Trials Group 320 Study Team.* N Engl J Med, 1997. **337**(11): p. 725-33.
34. Palella, F.J., Jr., et al., *Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. HIV Outpatient Study Investigators.* N Engl J Med, 1998. **338**(13): p. 853-60.
35. Deeks, S.G., et al., *HIV infection.* Nat Rev Dis Primers, 2015. **1**: p. 15035.
36. Barre-Sinoussi, F., A.L. Ross, and J.F. Delfraissy, *Past, present and future: 30 years of HIV research.* Nat Rev Microbiol, 2013. **11**(12): p. 877-83.
37. UNAIDS. *Fact sheet - Latest statistics on the status of the AIDS epidemic*. 2018.
38. Roberts, J.D., K. Bebenek, and T.A. Kunkel, *The accuracy of reverse transcriptase from HIV-1.* Science, 1988. **242**(4882): p. 1171-3.
39. Sharp, P.M. and B.H. Hahn, *The evolution of HIV-1 and the origin of AIDS.* Philos Trans R Soc Lond B Biol Sci, 2010. **365**(1552): p. 2487-94.
40. Pancera, M., et al., *Structure and immune recognition of trimeric pre-fusion HIV-1 Env.* Nature, 2014. **514**(7523): p. 455-61.
41. Turner, B.G. and M.F. Summers, *Structural biology of HIV.* J Mol Biol, 1999. **285**(1): p. 1-32.
42. Wilen, C.B., J.C. Tilton, and R.W. Doms, *HIV: cell binding and entry.* Cold Spring Harb Perspect Med, 2012. **2**(8).
43. Maartens, G., C. Celum, and S.R. Lewin, *HIV infection: epidemiology, pathogenesis, treatment, and prevention.* Lancet, 2014. **384**(9939): p. 258-71.
44. Arhel, N., *Revisiting HIV-1 uncoating.* Retrovirology, 2010. **7**: p. 96.
45. Li, X., et al., *Structural biology of retroviral DNA integration.* Virology, 2011. **411**(2): p. 194-205.

46. Chun, T.W., et al., *Early establishment of a pool of latently infected, resting CD4(+) T cells during primary HIV-1 infection.* Proc Natl Acad Sci U S A, 1998. **95**(15): p. 8869-73.
47. Engelman, A. and P. Cherepanov, *The structural biology of HIV-1: mechanistic and therapeutic insights.* Nat Rev Microbiol, 2012. **10**(4): p. 279-90.
48. Pettit, S.C., et al., *The p2 domain of human immunodeficiency virus type 1 Gag regulates sequential proteolytic processing and is required to produce fully infectious virions.* J Virol, 1994. **68**(12): p. 8017-27.
49. Ahmed, Z., et al., *The role of human dendritic cells in HIV-1 infection.* J Invest Dermatol, 2015. **135**(5): p. 1225-1233.
50. Salkowitz, J.R., et al., *Characterization of high-risk HIV-1 seronegative hemophiliacs.* Clin Immunol, 2001. **98**(2): p. 200-11.
51. Liu, R., et al., *Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection.* Cell, 1996. **86**(3): p. 367-77.
52. Tindall, B., et al., *Characterization of the acute clinical illness associated with human immunodeficiency virus infection.* Arch Intern Med, 1988. **148**(4): p. 945-9.
53. Little, S.J., et al., *Viral dynamics of acute HIV-1 infection.* J Exp Med, 1999. **190**(6): p. 841-50.
54. Levy, J.A., *HIV pathogenesis: 25 years of progress and persistent challenges.* Aids, 2009. **23**(2): p. 147-60.
55. Mellors, J.W., et al., *Prognosis in HIV-1 infection predicted by the quantity of virus in plasma.* Science, 1996. **272**(5265): p. 1167-70.
56. Silva Mde, O., et al., *Acute HIV infection with rapid progression to AIDS.* Braz J Infect Dis, 2010. **14**(3): p. 291-3.
57. Leon, A., et al., *Rate and predictors of progression in elite and viremic HIV-1 controllers.* Aids, 2016. **30**(8): p. 1209-20.
58. Okulicz, J.F., et al., *Clinical Outcomes of Elite Controllers, Viremic Controllers, and Long-Term Nonprogressors in the US Department of Defense HIV Natural History Study.* The Journal of Infectious Diseases, 2009. **200**(11): p. 1714-1723.
59. Autran, B., et al., *Positive effects of combined antiretroviral therapy on CD4+ T cell homeostasis and function in advanced HIV disease.* Science, 1997. **277**(5322): p. 112-6.
60. Cohen, M.S., et al., *Antiretroviral treatment of HIV-1 prevents transmission of HIV-1: where do we go from here?* Lancet, 2013. **382**(9903): p. 1515-24.
61. De Clercq, E., *The nucleoside reverse transcriptase inhibitors, nonnucleoside reverse transcriptase inhibitors, and protease inhibitors in the treatment of HIV infections (AIDS).* Adv Pharmacol, 2013. **67**: p. 317-58.
62. Tachedjian, G., et al., *Nonnucleoside reverse transcriptase inhibitors are chemical enhancers of dimerization of the HIV type 1 reverse transcriptase.* Proc Natl Acad Sci U S A, 2001. **98**(13): p. 7188-93.
63. Arts, E.J. and D.J. Hazuda, *HIV-1 antiretroviral drug therapy.* Cold Spring Harb Perspect Med, 2012. **2**(4): p. a007161.
64. Yarchoan, R., et al., *Administration of 3'-azido-3'-deoxythymidine, an inhibitor of HTLV-III/LAV replication, to patients with AIDS or AIDS-related complex.* Lancet, 1986. **1**(8481): p. 575-80.
65. Larder, B.A., G. Darby, and D.D. Richman, *HIV with reduced sensitivity to zidovudine (AZT) isolated during prolonged therapy.* Science, 1989. **243**(4899): p. 1731-4.
66. Stengel, R.F., *Mutation and control of the human immunodeficiency virus.* Math Biosci, 2008. **213**(2): p. 93-102.

67. Qian, K., S.L. Morris-Natschke, and K.-H. Lee, *HIV Entry Inhibitors and Their Potential in HIV Therapy.* Medicinal research reviews, 2009. **29**(2): p. 369-393.
68. Furtado, M.R., et al., *Persistence of HIV-1 transcription in peripheral-blood mononuclear cells in patients receiving potent antiretroviral therapy.* N Engl J Med, 1999. **340**(21): p. 1614-22.
69. Alexaki, A., Y. Liu, and B. Wigdahl, *Cellular Reservoirs of HIV-1 and their Role in Viral Persistence.* Current HIV research, 2008. **6**(5): p. 388-400.
70. Pierson, T., et al., *Characterization of chemokine receptor utilization of viruses in the latent reservoir for human immunodeficiency virus type 1.* J Virol, 2000. **74**(17): p. 7824-33.
71. Kumar, A., W. Abbas, and G. Herbein, *HIV-1 Latency in Monocytes/Macrophages.* Viruses, 2014. **6**(4): p. 1837-1860.
72. Freiberg, M.S., et al., *HIV infection and the risk of acute myocardial infarction.* JAMA Intern Med, 2013. **173**(8): p. 614-22.
73. De Wit, S., et al., *Incidence and risk factors for new-onset diabetes in HIV-infected patients: the Data Collection on Adverse Events of Anti-HIV Drugs (D:A:D) study.* Diabetes Care, 2008. **31**(6): p. 1224-9.
74. Brown, T.T. and R.B. Qaqish, *Antiretroviral therapy and the prevalence of osteopenia and osteoporosis: a meta-analytic review.* Aids, 2006. **20**(17): p. 2165-74.
75. Odden, M.C., et al., *Cystatin C level as a marker of kidney function in human immunodeficiency virus infection: the FRAM study.* Arch Intern Med, 2007. **167**(20): p. 2213-9.
76. Deeken, J.F., et al., *The rising challenge of non-AIDS-defining cancers in HIV-infected patients.* Clin Infect Dis, 2012. **55**(9): p. 1228-35.
77. Deeks, S.G., R. Tracy, and D.C. Douek, *Systemic Effects of Inflammation on Health during Chronic HIV Infection.* Immunity, 2013. **39**(4): p. 633-645.
78. Lapadula, G., et al., *Risk of clinical progression among patients with immunological nonresponse despite virological suppression after combination antiretroviral treatment.* Aids, 2013. **27**(5): p. 769-79.
79. Marin, B., et al., *Non-AIDS-defining deaths and immunodeficiency in the era of combination antiretroviral therapy.* Aids, 2009. **23**(13): p. 1743-53.
80. van Lelyveld, S.F., et al., *Long-term complications in patients with poor immunological recovery despite virological successful HAART in Dutch ATHENA cohort.* Aids, 2012. **26**(4): p. 465-74.
81. Gaardbo, J.C., et al., *Incomplete immune recovery in HIV infection: mechanisms, relevance for clinical care, and possible solutions.* Clin Dev Immunol, 2012. **2012**: p. 670957.
82. Teixeira, L., et al., *Poor CD4 T cell restoration after suppression of HIV-1 replication may reflect lower thymic function.* Aids, 2001. **15**(14): p. 1749-56.
83. Brenchley, J.M., et al., *Microbial translocation is a cause of systemic immune activation in chronic HIV infection.* Nat Med, 2006. **12**(12): p. 1365-71.
84. Manjati, T., B. Nkambule, and H. Ipp, *Immune activation is associated with decreased thymic function in asymptomatic, untreated HIV-infected individuals.* Southern African Journal of HIV Medicine, 2016. **17**(1): p. 445.
85. Borges, Á.H., et al., *Interleukin 6 Is a Stronger Predictor of Clinical Events Than High-Sensitivity C-Reactive Protein or D-Dimer During HIV Infection.* The Journal of Infectious Diseases, 2016. **214**(3): p. 408-416.
86. Neuhaus, J., et al., *Markers of inflammation, coagulation, and renal function are elevated in adults with HIV infection.* J Infect Dis, 2010. **201**(12): p. 1788-95.

87. Sandler, N.G. and D.C. Douek, *Microbial translocation in HIV infection: causes, consequences and treatment opportunities.* Nat Rev Microbiol, 2012. **10**(9): p. 655-66.
88. Burdo, T.H., et al., *Soluble CD163 made by monocyte/macrophages is a novel marker of HIV activity in early and chronic infection prior to and after anti-retroviral therapy.* J Infect Dis, 2011. **204**(1): p. 154-63.
89. Veazey, R.S., et al., *Gastrointestinal tract as a major site of CD4+ T cell depletion and viral replication in SIV infection.* Science, 1998. **280**(5362): p. 427-31.
90. Monroe, K.M., et al., *IFI16 DNA sensor is required for death of lymphoid CD4 T cells abortively infected with HIV.* Science, 2014. **343**(6169): p. 428-32.
91. Doitsh, G., et al., *Cell death by pyroptosis drives CD4 T-cell depletion in HIV-1 infection.* Nature, 2014. **505**(7484): p. 509-14.
92. Brenchley, J.M., et al., *Differential Th17 CD4 T-cell depletion in pathogenic and nonpathogenic lentiviral infections.* Blood, 2008. **112**(7): p. 2826-35.
93. Gessani, S., et al., *Enhanced production of LPS-induced cytokines during differentiation of human monocytes to macrophages. Role of LPS receptors.* J Immunol, 1993. **151**(7): p. 3758-66.
94. Jiang, W., et al., *Plasma levels of bacterial DNA correlate with immune activation and the magnitude of immune restoration in persons with antiretroviral-treated HIV infection.* J Infect Dis, 2009. **199**(8): p. 1177-85.
95. Caradonna, L., et al., *Enteric bacteria, lipopolysaccharides and related cytokines in inflammatory bowel disease: biological and clinical significance.* J Endotoxin Res, 2000. **6**(3): p. 205-14.
96. Lassenius, M.I., et al., *Bacterial Endotoxin Activity in Human Serum Is Associated With Dyslipidemia, Insulin Resistance, Obesity, and Chronic Inflammation.* Diabetes Care, 2011. **34**(8): p. 1809-1815.
97. Dervenis, C., D. Smailis, and E. Hatzitheoklitos, *Bacterial translocation and its prevention in acute pancreatitis.* J Hepatobiliary Pancreat Surg, 2003. **10**(6): p. 415-8.
98. Kanneganti, T.D., M. Lamkanfi, and G. Nunez, *Intracellular NOD-like receptors in host defense and disease.* Immunity, 2007. **27**(4): p. 549-59.
99. Mellor, A.L. and D.H. Munn, *IDO expression by dendritic cells: tolerance and tryptophan catabolism.* Nat Rev Immunol, 2004. **4**(10): p. 762-74.
100. Munn, D.H., et al., *GCN2 kinase in T cells mediates proliferative arrest and anergy induction in response to indoleamine 2,3-dioxygenase.* Immunity, 2005. **22**(5): p. 633-42.
101. Favre, D., et al., *Tryptophan catabolism by indoleamine 2,3-dioxygenase 1 alters the balance of TH17 to regulatory T cells in HIV disease.* Sci Transl Med, 2010. **2**(32): p. 32ra36.
102. Appay, V. and D. Sauce, *Immune activation and inflammation in HIV-1 infection: causes and consequences.* J Pathol, 2008. **214**(2): p. 231-41.
103. Erlandson, K.M. and T.B. Campbell, *Inflammation in Chronic HIV Infection: What Can We Do?* The Journal of Infectious Diseases, 2015. **212**(3): p. 339-342.
104. Sandoval-Montes, C. and L. Santos-Argumedo, *CD38 is expressed selectively during the activation of a subset of mature T cells with reduced proliferation but improved potential to produce cytokines.* J Leukoc Biol, 2005. **77**(4): p. 513-21.
105. Rah, S.-Y. and U.-H. Kim, *CD38-mediated Ca(2+) signaling contributes to glucagon-induced hepatic gluconeogenesis.* Scientific Reports, 2015. **5**: p. 10741.
106. Zubiaur, M., et al., *The CD3-gamma delta epsilon transducing module mediates CD38-induced protein-tyrosine kinase and mitogen-activated protein kinase activation in Jurkat T cells.* J Biol Chem, 1999. **274**(29): p. 20633-42.

107. Imamichi, H., et al., *The CD8+ HLA-DR+ T cells expanded in HIV-1 infection are qualitatively identical to those from healthy controls.* Eur J Immunol, 2012. **42**(10): p. 2608-20.
108. Thomas, M.L., *The leukocyte common antigen family.* Annu Rev Immunol, 1989. **7**: p. 339-69.
109. Sallusto, F., J. Geginat, and A. Lanzavecchia, *Central memory and effector memory T cell subsets: function, generation, and maintenance.* Annu Rev Immunol, 2004. **22**: p. 745-63.
110. Tian, Y., et al., *Unique phenotypes and clonal expansions of human CD4 effector memory T cells re-expressing CD45RA.* Nat Commun, 2017. **8**(1): p. 1473.
111. Denoeud, J. and M. Moser, *Role of CD27/CD70 pathway of activation in immunity and tolerance.* J Leukoc Biol, 2011. **89**(2): p. 195-203.
112. Mahnke, Y.D., et al., *The who's who of T-cell differentiation: human memory T-cell subsets.* Eur J Immunol, 2013. **43**(11): p. 2797-809.
113. Pantaleo, G., et al., *Evidence for rapid disappearance of initially expanded HIV-specific CD8+ T cell clones during primary HIV infection.* Proc Natl Acad Sci U S A, 1997. **94**(18): p. 9848-53.
114. Kalams, S.A., et al., *Association between virus-specific cytotoxic T-lymphocyte and helper responses in human immunodeficiency virus type 1 infection.* J Virol, 1999. **73**(8): p. 6715-20.
115. Betts, M.R., et al., *HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells.* Blood, 2006. **107**(12): p. 4781-9.
116. Demers, K.R., M.A. Reuter, and M.R. Betts, *CD8(+) T-cell effector function and transcriptional regulation during HIV pathogenesis.* Immunological reviews, 2013. **254**(1): p. 190-206.
117. Letterio, J.J. and A.B. Roberts, *Regulation of immune responses by TGF-beta.* Annu Rev Immunol, 1998. **16**: p. 137-61.
118. Li, M.O., et al., *Transforming growth factor-beta regulation of immune responses.* Annu Rev Immunol, 2006. **24**: p. 99-146.
119. Pitcher, C.J., et al., *HIV-1-specific CD4+ T cells are detectable in most individuals with active HIV-1 infection, but decline with prolonged viral suppression.* Nat Med, 1999. **5**(5): p. 518-25.
120. Mohan, T., et al., *Current understanding of HIV-1 and T-cell adaptive immunity: Progress to date.* Microbial Pathogenesis, 2014. **73**: p. 60-69.
121. Wykes, M.N. and S.R. Lewin, *Immune checkpoint blockade in infectious diseases.* Nat Rev Immunol, 2018. **18**(2): p. 91-104.
122. Greenwald, R.J., G.J. Freeman, and A.H. Sharpe, *THE B7 FAMILY REVISITED.* Annual Review of Immunology, 2005. **23**(1): p. 515-548.
123. Sharpe, A.H. and K.E. Pauken, *The diverse functions of the PD1 inhibitory pathway.* Nat Rev Immunol, 2018. **18**(3): p. 153-167.
124. Zhang, X., et al., *Structural and functional analysis of the costimulatory receptor programmed death-1.* Immunity, 2004. **20**(3): p. 337-47.
125. Sheppard, K.A., et al., *PD-1 inhibits T-cell receptor induced phosphorylation of the ZAP70/CD3zeta signalosome and downstream signaling to PKCtheta.* FEBS Lett, 2004. **574**(1-3): p. 37-41.
126. Okazaki, T., Y. Iwai, and T. Honjo, *New regulatory co-receptors: inducible co-stimulator and PD-1.* Curr Opin Immunol, 2002. **14**(6): p. 779-82.
127. Richter, K., P. Agnellini, and A. Oxenius, *On the role of the inhibitory receptor LAG-3 in acute and chronic LCMV infection.* Int Immunol, 2010. **22**(1): p. 13-23.

128. Anderson, A.C., N. Joller, and V.K. Kuchroo, *Lag-3, Tim-3, and TIGIT co-inhibitory receptors with specialized functions in immune regulation.* Immunity, 2016. **44**(5): p. 989-1004.
129. Hannier, S., et al., *CD3/TCR complex-associated lymphocyte activation gene-3 molecules inhibit CD3/TCR signaling.* J Immunol, 1998. **161**(8): p. 4058-65.
130. Zhu, C., et al., *The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity.* Nat Immunol, 2005. **6**(12): p. 1245-52.
131. Huang, Y.H., et al., *CEACAM1 regulates TIM-3-mediated tolerance and exhaustion.* Nature, 2015. **517**(7534): p. 386-90.
132. Joller, N., et al., *TIGIT has T cell intrinsic inhibitory functions.* Journal of immunology (Baltimore, Md. : 1950), 2011. **186**(3): p. 1338-1342.
133. Stanietsky, N., et al., *The interaction of TIGIT with PVR and PVRL2 inhibits human NK cell cytotoxicity.* Proceedings of the National Academy of Sciences, 2009. **106**(42): p. 17858-17863.
134. Tu, T.C., et al., *CD160 is essential for NK-mediated IFN-γ production.* The Journal of Experimental Medicine, 2015. **212**(3): p. 415-429.
135. Cheung, T.C., et al., *Evolutionarily divergent herpesviruses modulate T cell activation by targeting the herpesvirus entry mediator cosignaling pathway.* Proc Natl Acad Sci U S A, 2005. **102**(37): p. 13218-23.
136. Desai, S. and A. Landay, *Early immune senescence in HIV disease.* Curr HIV/AIDS Rep, 2010. **7**(1): p. 4-10.
137. Henson, S.M. and A.N. Akbar, *KLRG1—more than a marker for T cell senescence.* Age, 2009. **31**(4): p. 285-291.
138. Joshi, N.S., et al., *Inflammation Directs Memory Precursor and Short-Lived Effector CD8+ T Cell Fates via the Graded Expression of T-bet Transcription Factor.* Immunity, 2007. **27**(2): p. 281-295.
139. Chew, G.M., et al., *TIGIT Marks Exhausted T Cells, Correlates with Disease Progression, and Serves as a Target for Immune Restoration in HIV and SIV Infection.* PLoS Pathogens, 2016. **12**(1): p. e1005349.
140. Dougall, W.C., et al., *TIGIT and CD96: new checkpoint receptor targets for cancer immunotherapy.* Immunol Rev, 2017. **276**(1): p. 112-120.
141. Seung, E., et al., *PD-1 blockade in chronically HIV-1-infected humanized mice suppresses viral loads.* PLoS One, 2013. **8**(10): p. e77780.
142. Iwai, Y., et al., *Cancer immunotherapies targeting the PD-1 signaling pathway.* J Biomed Sci, 2017. **24**(1): p. 26.
143. Koyama, S., et al., *Adaptive resistance to therapeutic PD-1 blockade is associated with upregulation of alternative immune checkpoints.* Nat Commun, 2016. **7**: p. 10501.
144. Porichis, F., et al., *Responsiveness of HIV-specific CD4 T cells to PD-1 blockade.* Blood, 2011. **118**(4): p. 965-974.
145. Briceño, O., et al., *Gut Homing CD4+ and CD8+ T-Cell Frequencies in HIV Infected Individuals on Antiretroviral Treatment.* PLoS ONE, 2016. **11**(11): p. e0166496.
146. Hait, S.H., et al., *Conservation of the alpha4beta7 lymphocyte homing receptor in HIV-infected patients with distinct transmission routes and disease progression profiles.* AIDS Res Hum Retroviruses, 2014. **30**(5): p. 493-7.
147. Jaroszeski, M.J. and G. Radcliff, *Fundamentals of flow cytometry.* Molecular Biotechnology, 1999. **11**(1): p. 37-53.
148. BioRad. *Flow cytometry Basic Guide*.
149. *Flow Cytometry*.

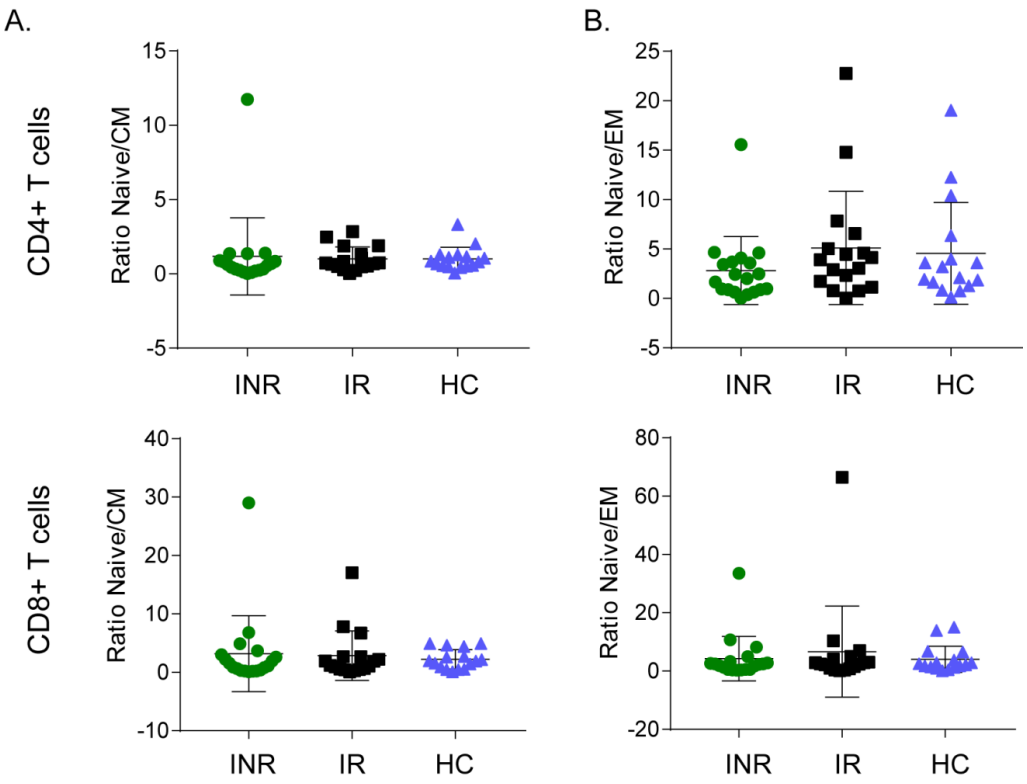
150. Maciorowski, Z., P.K. Chattopadhyay, and P. Jain, *Basic Multicolor Flow Cytometry.* Curr Protoc Immunol, 2017. **117**: p. 5.4.1-5.4.38.
151. Scientific, T. *Dynabeads Cell Isolation and Expansion Support—Getting Started.* 2018.
152. DeNucci, C.C., et al., *CONTROL OF α4β7 INTEGRIN EXPRESSION AND CD4 T CELL HOMING BY THE β1 INTEGRIN SUBUNIT.* Journal of immunology (Baltimore, Md. : 1950), 2010. **184**(5): p. 2458-2467.
153. Battegay, M., et al., *Immunological recovery and antiretroviral therapy in HIV-1 infection.* Lancet Infect Dis, 2006. **6**(5): p. 280-7.
154. Kaufmann, G.R., et al., *Characteristics, determinants, and clinical relevance of CD4 T cell recovery to <500 cells/microL in HIV type 1-infected individuals receiving potent antiretroviral therapy.* Clin Infect Dis, 2005. **41**(3): p. 361-72.
155. Serrano-Villar, S., et al., *HIV-infected individuals with low CD4/CD8 ratio despite effective antiretroviral therapy exhibit altered T cell subsets, heightened CD8+ T cell activation, and increased risk of non-AIDS morbidity and mortality.* PLoS Pathog, 2014. **10**(5): p. e1004078.
156. Kaufmann, G.R., et al., *CD4 T-lymphocyte recovery in individuals with advanced HIV-1 infection receiving potent antiretroviral therapy for 4 years: the Swiss HIV Cohort Study.* Arch Intern Med, 2003. **163**(18): p. 2187-95.
157. Stiksrud, B., et al., *Plasma IP-10 Is Increased in Immunological NonResponders and Associated With Activated Regulatory T Cells and Persisting Low CD4 Counts.* J Acquir Immune Defic Syndr, 2016. **73**(2): p. 138-48.
158. Massanella, M., et al., *CD4 T-cell hyperactivation and susceptibility to cell death determine poor CD4 T-cell recovery during suppressive HAART.* Aids, 2010. **24**(7): p. 959-68.
159. Landay, A., et al., *Evidence of ongoing immune reconstitution in subjects with sustained viral suppression following 6 years of lopinavir-ritonavir treatment.* Clin Infect Dis, 2007. **44**(5): p. 749-54.
160. Negredo, E., et al., *Nadir CD4 T cell count as predictor and high CD4 T cell intrinsic apoptosis as final mechanism of poor CD4 T cell recovery in virologically suppressed HIV-infected patients: clinical implications.* Clin Infect Dis, 2010. **50**(9): p. 1300-8.
161. Day, C.L., et al., *PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression.* Nature, 2006. **443**(7109): p. 350-4.
162. Tauriainen, J., et al., *Perturbed CD8(+) T cell TIGIT/CD226/PVR axis despite early initiation of antiretroviral treatment in HIV infected individuals.* Sci Rep, 2017. **7**: p. 40354.
163. Tian, X., et al., *The upregulation of LAG-3 on T cells defines a subpopulation with functional exhaustion and correlates with disease progression in HIV-infected subjects.* J Immunol, 2015. **194**(8): p. 3873-82.
164. Fromentin, R., et al., *CD4+ T Cells Expressing PD-1, TIGIT and LAG-3 Contribute to HIV Persistence during ART.* PLoS Pathog, 2016. **12**(7): p. e1005761.
165. Jones, R.B., et al., *Tim-3 expression defines a novel population of dysfunctional T cells with highly elevated frequencies in progressive HIV-1 infection.* The Journal of Experimental Medicine, 2008. **205**(12): p. 2763-2779.
166. Bushnell, T. *Importance of Antibody Titration in Flow cytometry*.
167. Nguyen, R., et al., *Quantifying Spillover Spreading for Comparing Instrument Performance and Aiding in Multicolor Panel Design.* Cytometry. Part A : the journal of the International Society for Analytical Cytology, 2013. **83**(3): p. 306-315.
168. Phillips, A.N., J. Neaton, and J.D. Lundgren, *The role of HIV in serious diseases other than AIDS.* Aids, 2008. **22**(18): p. 2409-18.

169. Lee, S.A., et al., *Impact of HIV on CD8+ T cell CD57 expression is distinct from that of CMV and aging.* PLoS One, 2014. **9**(2): p. e89444.
170. Tavenier, J., et al., *Immunosenescence of the CD8(+) T cell compartment is associated with HIV-infection, but only weakly reflects age-related processes of adipose tissue, metabolism, and muscle in antiretroviral therapy-treated HIV-infected patients and controls.* BMC Immunology, 2015. **16**: p. 72.
171. Cicala, C., et al., *The integrin alpha4beta7 forms a complex with cell-surface CD4 and defines a T-cell subset that is highly susceptible to infection by HIV-1.* Proc Natl Acad Sci U S A, 2009. **106**(49): p. 20877-82.
172. Wang, X., et al., *Monitoring alpha4beta7 integrin expression on circulating CD4+ T cells as a surrogate marker for tracking intestinal CD4+ T-cell loss in SIV infection.* Mucosal Immunol, 2009. **2**(6): p. 518-26.
173. Sivro, A., et al., *Integrin alpha4beta7 expression on peripheral blood CD4(+) T cells predicts HIV acquisition and disease progression outcomes.* Sci Transl Med, 2018. **10**(425).
174. Girard, A., et al., *Brief Report: A High Rate of β7(+) Gut-Homing Lymphocytes in HIV-Infected Immunological Nonresponders is Associated With Poor CD4 T-Cell Recovery During Suppressive HAART.* Journal of Acquired Immune Deficiency Syndromes (1999), 2016. **72**(3): p. 259-265.
175. Ciccone, E.J., et al., *Cycling of Gut Mucosal CD4+ T Cells Decreases after Prolonged Anti-Retroviral Therapy and is Associated with Plasma LPS Levels.* Mucosal immunology, 2010. **3**(2): p. 172-181.
176. Jekle, A., et al., *In vivo evolution of human immunodeficiency virus type 1 toward increased pathogenicity through CXCR4-mediated killing of uninfected CD4 T cells.* J Virol, 2003. **77**(10): p. 5846-54.
177. Finkel, T.H., et al., *Apoptosis occurs predominantly in bystander cells and not in productively infected cells of HIV- and SIV-infected lymph nodes.* Nat Med, 1995. **1**(2): p. 129-34.
178. Doitsh, G., et al., *Abortive HIV Infection Mediates CD4 T-Cell Depletion and Inflammation in Human Lymphoid Tissue.* Cell, 2010. **143**(5): p. 789-801.
179. Li, J., et al., *PD-1/SHP-2 inhibits Tc1/Th1 phenotypic responses and the activation of T cells in the tumor microenvironment.* Cancer Res, 2015. **75**(3): p. 508-518.
180. Li, C., et al., *Peptide Blocking of PD-1/PD-L1 Interaction for Cancer Immunotherapy.* Cancer Immunol Res, 2018. **6**(2): p. 178-188.
181. Velu, V., et al., *Role of PD-1 co-inhibitory pathway in HIV infection and potential therapeutic options.* Retrovirology, 2015. **12**: p. 14.
182. Parry, R.V., et al., *CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms.* Mol Cell Biol, 2005. **25**(21): p. 9543-53.
183. Palmer, B.E., et al., *In vivo blockade of the PD-1 receptor suppresses HIV-1 viral loads and improves CD4+ T cell levels in humanized mice.* Journal of immunology (Baltimore, Md. : 1950), 2013. **190**(1): p. 211-219.
184. Patsoukis, N., et al., *PD-1 increases PTEN phosphatase activity while decreasing PTEN protein stability by inhibiting casein kinase 2.* Mol Cell Biol, 2013. **33**(16): p. 3091-8.
185. Kalamasz, D., et al., *Optimization of human T-cell expansion ex vivo using magnetic beads conjugated with anti-CD3 and Anti-CD28 antibodies.* J Immunother, 2004. **27**(5): p. 405-18.
186. Maecker, H.T. and J. Trotter, *Flow cytometry controls, instrument setup, and the determination of positivity.* Cytometry A, 2006. **69**(9): p. 1037-42.

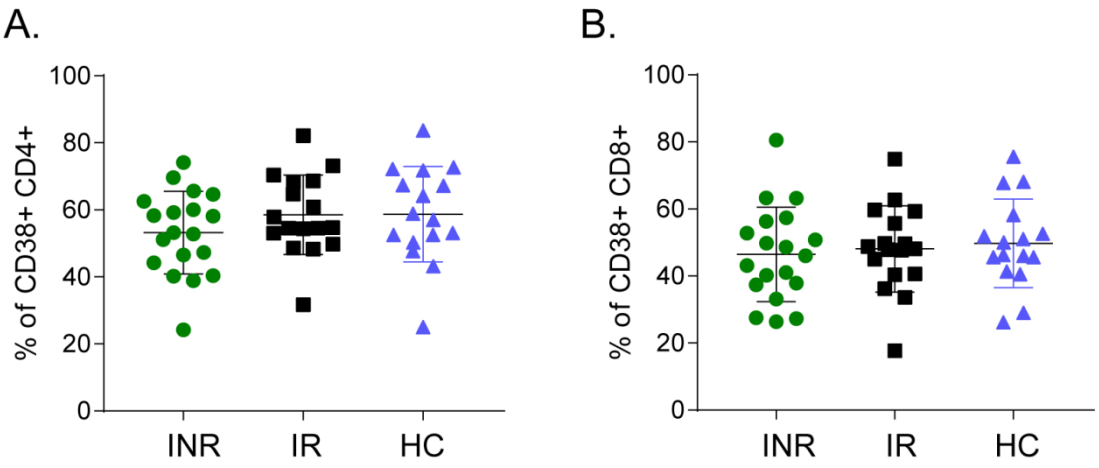
187. Wang, L., et al., *Standardization of cryopreserved peripheral blood mononuclear cells through a resting process for clinical immunomonitoring--Development of an algorithm.* Cytometry A, 2016. **89**(3): p. 246-58.

7 Appendix

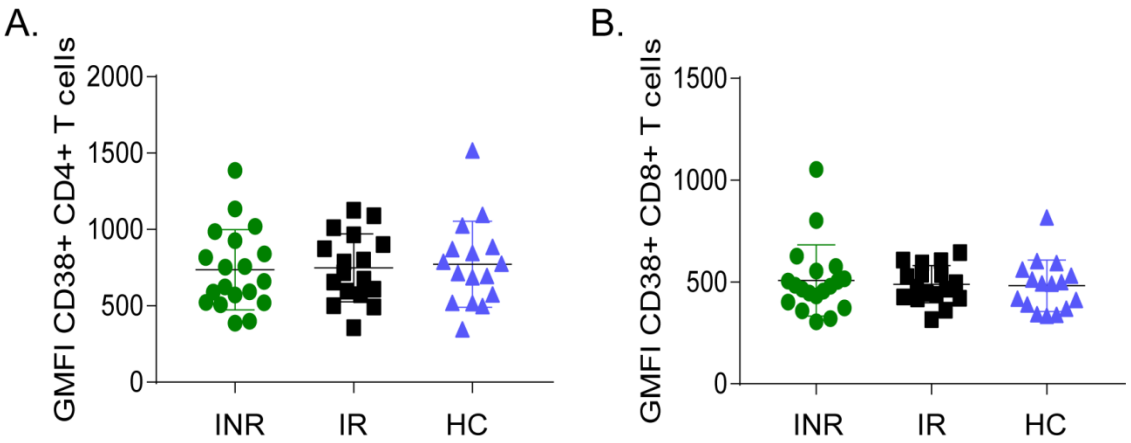
Supplement figure 1. Gating strategy based on FMO controls.



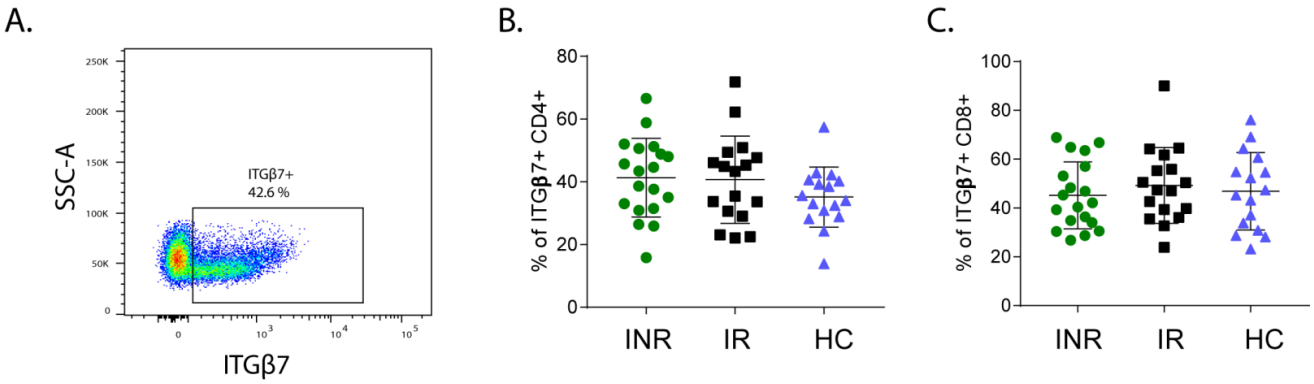
Supplementary Figure 2. Similar ratios of naive/CM and naive/EM T cells between the groups. *PBMCs were stained with Abs against HLA-DR and CD38 in order to determine the ratio between naive/CM T cells and naive/EM T cells. A) The ratio between naive and CM T cells compared between the groups are seen for CD4+ T cells (upper image) and CD8+ T cells (lower image). B) The ratio between naive and EM T cells for CD4+ T cells (upper image) and CD8+ T cells (lower image). Lines indicate the mean ± SD.*



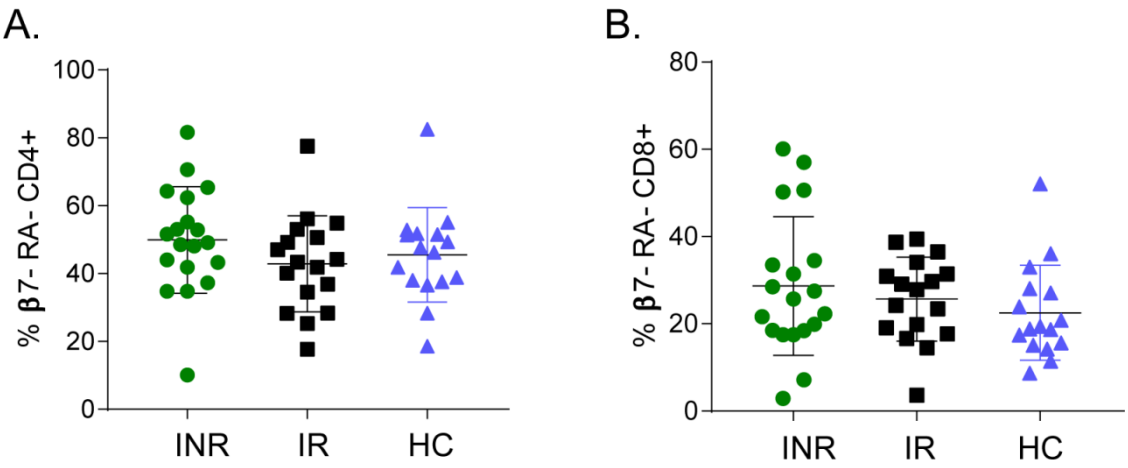
Supplementary Figure 3. Similar expression of CD38+ T cells. *PBMCs were stained with anti-CD38. Calculated percentages of CD38+ CD4+ T cells (A), and for CD8+ T cells (B). Lines indicate the mean ± SD.*



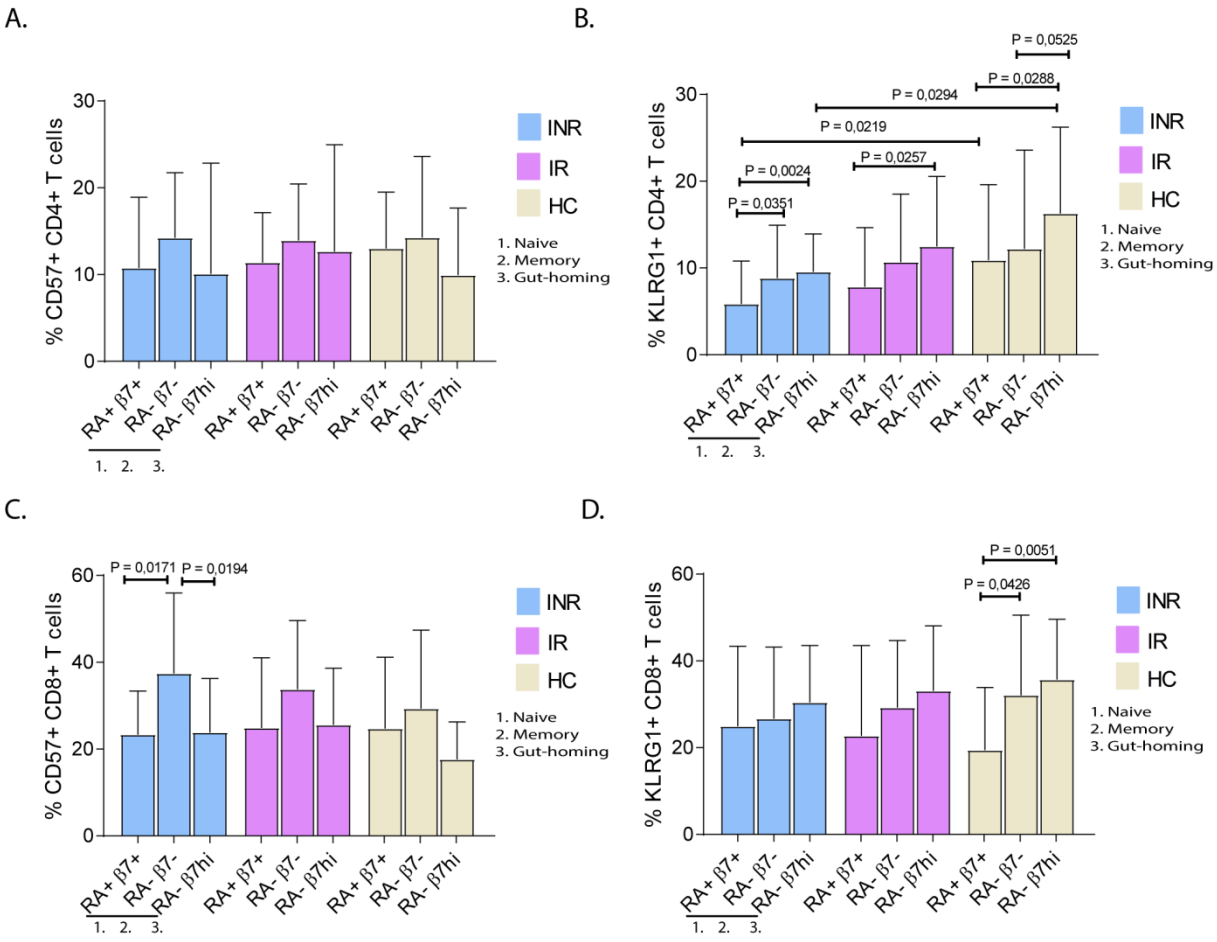
Supplementary Figure 4. Geometric mean fluorescence intensity (GMFI) of CD38 expression. *GMFI of CD38 is shown for CD4+ T cells (A) and CD8+ T cells (B). Lines indicate the mean ± SD.*



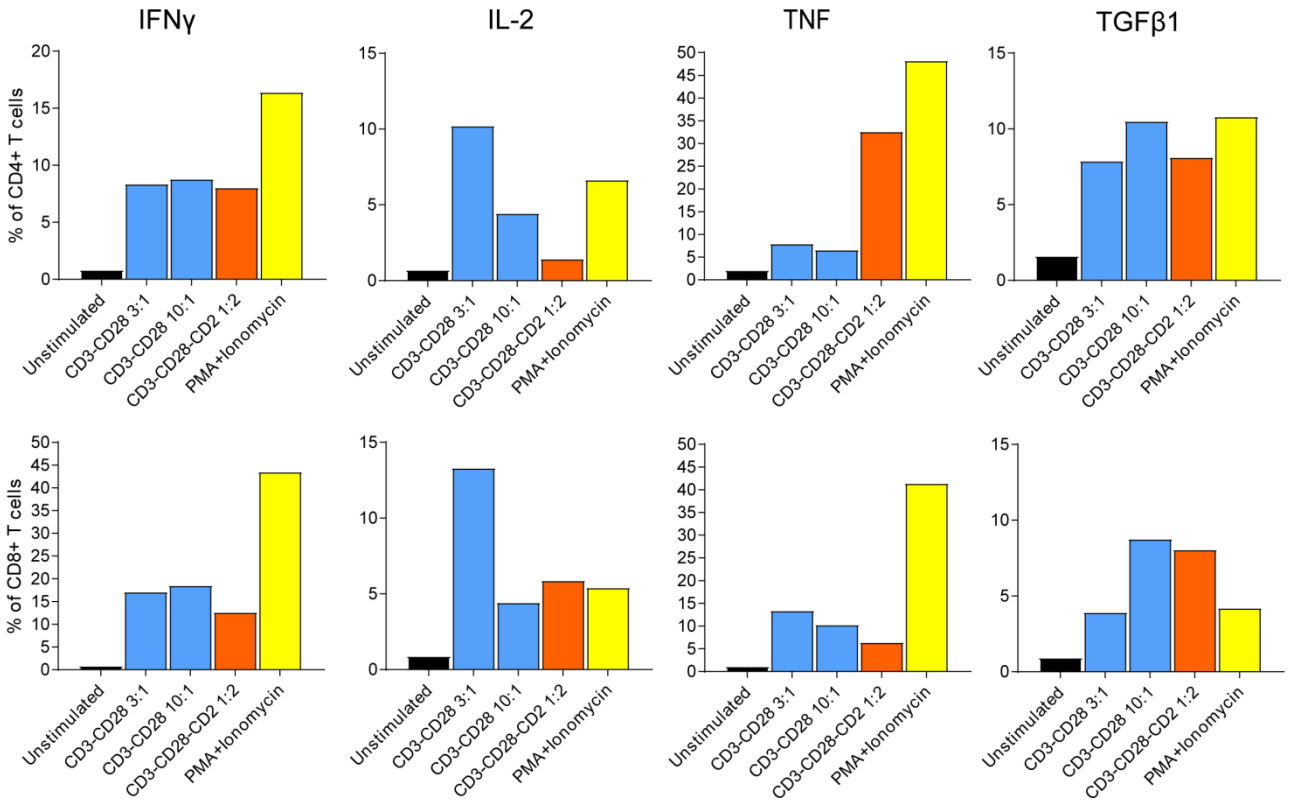
Supplementary Figure 5. Similar expression of ITGβ7+ T cells. *PBMCs were stained with anti-ITGβ7. Calculated percentages of ITGβ7+ CD4+ T cells (A), and CD8+ T cells (B). Lines indicate the mean ± SD.*



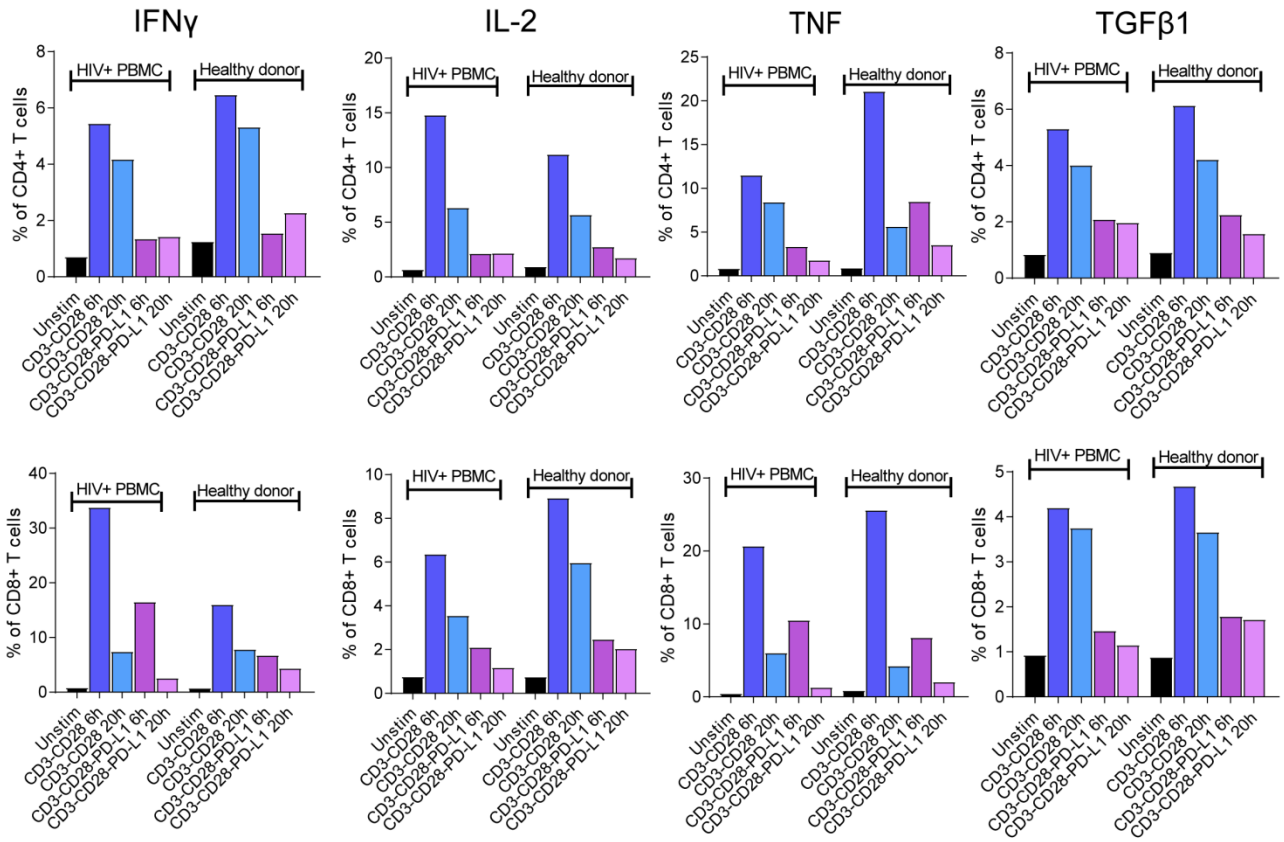
Supplementary Figure 6. Similar expression of β7- RA- T cells. *Calculated percentages of CD4+ memory T cells (CD45RA- ITGβ7-) (A), and for CD8+ memory T cells (CD45RA- ITGβ7-) (B). Lines indicate the mean ± SD.*



Supplementary Figure 7. Expression of CD57 and KLRG1 in relation to gut-homing T cells. *In blue are percentages from INR patients, in purple IR patients and in beige the HC group. Comparison of the percentages of (1) naive (CD45RA+ ITGβ7+), (2) memory (CD45RA- ITGβ7-) and (3) gut-homing (CD45RA- ITGβ7high) CD4+ T cells expressing CD57 (A), and KLRG1 (B). Comparison of the percentages of (1) naive, (2) memory, and (3) gut-homing CD8+ T cells expressing CD57 (C), and KLRG1 (D). P values were calculated with non-parametric Mann-Whitney U- test. Lines indicate the mean ± SD*



Supplement figure 8. Coating Abs on Dynabeads, Miltenyi beads and cells stimulated with PMA + ionomycin all gave sufficient cytokine production. *PBMCs from healthy blood donor were stimulated with Dynabeads, Miltenyi beads or PMA + ionomycin. Two ratio beads to cell ratios were tested for the Dynabeads. Cells were stimulated for 20 hours before fixated and permeabilized. The cells were stained with Abs against IFNγ, IL-2, TGFβand TNFα and analysed on the flow cytometer. Unstimulated cells are shown in black, cells stimulated with Dynabeads in blue, cells stimulated with Miltenyi beads on orange, and cells stimulated with PMA + ionomycin in yellow. The upper part of the figure shows the cytokine production for CD4+ T cells, and the lower part for the CD8+ T cells.*



Supplementary Figure 9. Six hours stimulation with beads gave the highest cytokine production and significant inhibition for both healthy and HIV+ PBMCs. *HIV+ PBMCs and PBMCs from a healthy donor were stimulated with beads coupled with anti-CD3/CD28 or anti-CD3/CD28/PD-L1 for 6 or 20 hours before the cells were fixated and permeabilized. The cells were stained at the same time the next day with the appropriate Abs. Unstimulated cells are shown in black, cells stimulated with anti-CD3/CD28 in blue and cells stimulated with anti-CD3/CD28/PD-L1 in purple. The upper part of the figure shows the cytokine production for CD4+ T cells, and the lower part for the CD8+ T cells.*