Immune activation and regulation in chronic HIV infection: Implications for immune reconstitution and therapeutic strategies

by

Christian Prebensen

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Institute of Clinical Medicine
Faculty of Medicine
The University of Oslo
&
Department of Infectious Diseases
Oslo University Hospital

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Abbreviations

AIDS	acquired immune deficiency syndrome
APC:	antigen-presenting cell
ART:	antiretroviral therapy
bNAbs	broadly neutralising antibodies
cAMP:	cyclic adenosine monophosphate
CAT:	calibrated automated thrombogram
CFSE:	carboxyfluorescein diacetate succinimidyl ester
COX:	cyclooxygenase
CTL:	cytotoxic T lymphocyte
CCR5	C-C chemokine receptor 5
DC:	dendritic cell
ELISA:	enzyme-linked immunosorbent assay
FMO:	fluorescence-minus-one
FTR:	functional T cell regulation
GALT:	gut-associated lymphoid tissue
HDACi:	histone deacetylase inhibitor
HIV	human immunodeficiency virus
HLA:	human leukocyte antigen
IDO:	indoleamine 2,3-dioxygenase
IFN-γ:	interferon gamma
IL:	interleukin
INR:	immunological non-responder
IP-10:	interferon gamma-inducible protein 10
KT ratio:	kynurenine/tryptophan ratio
LC-MS:	liquid chromatography – tandem mass spectrometry
LCMV	lymphocytic choriomeningitis virus
MIP-1β:	macrophage inflammatory protein 1 beta
NHP:	non-human primate
NSAID	non-steroidal anti-inflammatory drug
TCR:	T cell receptor
PD-1:	programmed death 1
PGE_2:	prostaglandin E_2
PKA:	protein kinase A
SIV:	simian immunodeficiency virus
TGF-β:	transforming growth factor beta
TLR:	Toll-like receptor
TNF-α:	tumour necrosis factor a
Treg:	regulatory T cell

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Paper II: Regulation of Gag- and Env-Specific $CD8^+$ T Cell Responses in ART-Naïve HIV-Infected Patients: Potential Implications for Individualized Immuno-therapy.

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PLoS One. 2016;11(4): e0153849

Paper III: Immune Activation and HIV-Specific T cell Responses are Modulated by a Cyclooxygenase-2 Inhibitor in Untreated HIV-Infected Individuals: An Exploratory Clinical Trial.

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Submitted manuscript

1. Introduction

Over the past three and a half decades, the HIV/AIDS pandemic has caused immense human suffering, but also sparked a vigorous response from the scientific community. Fundamental insights into how HIV replicates in the body have facilitated the development of highly effective therapy, which is increasingly available globally. However, this antiretroviral therapy (ART) is not curative, and even treated HIV infection is associated with an increased incidence of non-AIDS-related diseases. It is now clear that the host immune response to HIV infection is not only incapable of clearing the virus, but also responsible for excessive collateral damage throughout the body. Furthermore, this inflammatory condition persists to some degree despite suppression of viral replication by ART, and may impair the function and regeneration of the immune system of treated patients.

In order to further improve the health and prognosis of HIV-infected patients, immunotherapeutic strategies to attenuate harmful HIV-associated immune activation and inflammation are warranted. Moreover, there is currently a renewed optimism about the prospects for a functional HIV cure, that is, virological remission in the absence of ART. To this end, patients' compromised HIV-specific immunity will likely need boosting by immunotherapeutic approaches.

This thesis encompasses investigations into HIV-associated immune activation, inflammation and immune regulation, with an emphasis on their potential implications for immune reconstitution on ART and response to immunotherapy. The following review of the field seeks to contextualise the scientific work presented herein, and introduces certain key concepts.

1.1 History and epidemiology

1.1.1 Emergence of HIV

On June 5, 1981, the US Centers for Disease Control published its Morbidity and Mortality Weekly Report, describing the puzzling case of Pneumocystis pneumonia in five previously healthy homosexual men in Los Angeles [1]. Similar cases were soon

reported from New York, San Francisco and elsewhere: patients presenting with Pneumocystis, Kaposi's sarcoma, mucosal candidiasis, CMV disease and apparent defects in cellular immunity [2, 3]. By the following year, the term acquired immune deficiency syndrome (AIDS) had been coined to describe this clearly communicable condition [4]. The responsible retrovirus was isolated in 1983, initially referred to as lymphadenopathy-associated virus (LAV) and human t-lymphotropic virus type III (HTLV-III) [5, 6]. In 1986 the virus received its current moniker, human immunodeficiency virus (HIV) [7]. However, it is more precisely termed HIV-1, as a related retrovirus was discovered in West African patients with AIDS the same year [8], and has since been known as HIV-2. HIV-1, the virus responsible for the global pandemic, is the subject of this thesis, and will from here on simply be termed HIV.

Chimpanzees of West-Central Africa have been established as the natural reservoir of HIV infection, harbouring the most closely related simian immunodeficiency virus (SIV_{CPZ}) [9]. Phylogenetic analyses comparing the two viruses suggest a string of separate transmission events from ape to human during the late 19th and early 20th centuries [10, 11]. The first human HIV isolate was identified in plasma from an adult male from Leopoldville, Belgian Congo (now Kinshasa, Democratic Republic of Congo) in 1959 [12]. The first retrospectively confirmed case of AIDS was an adolescent male from Missouri in 1969 [13], and in Europe, a Norwegian sailor and his family who died in 1976 [14].

1.1.2 Epidemiology

UNAIDS estimates that, as of 2015, 78 million people had been infected with HIV, and 35 million had died of AIDS-related disease. At that time, some 37 million people were estimated to be living with HIV infection, of whom 25.8 million were in sub-Saharan Africa. An estimated 1.1 million people died of AIDS-related causes in 2015, a reduction of around 45% from the peak year 2005, in which 2 million died [15]. This made HIV the sixth leading cause of death worldwide [16].

Antiretroviral therapy (ART), increasingly available since the end of the 1990s, has changed the nature of HIV disease. From a near-certain death sentence, HIV infection has become a manageable chronic condition, but only for those who can and do access treatment. In 2015, 17 million, or 46% of HIV-infected people, were receiving ART, and 49% of infected children were being treated [15]. Furthermore, despite a continuous scale-up of ART, the number of patients initiating treatment still lags behind the rate of new infections, which was 2.1 million in 2015.

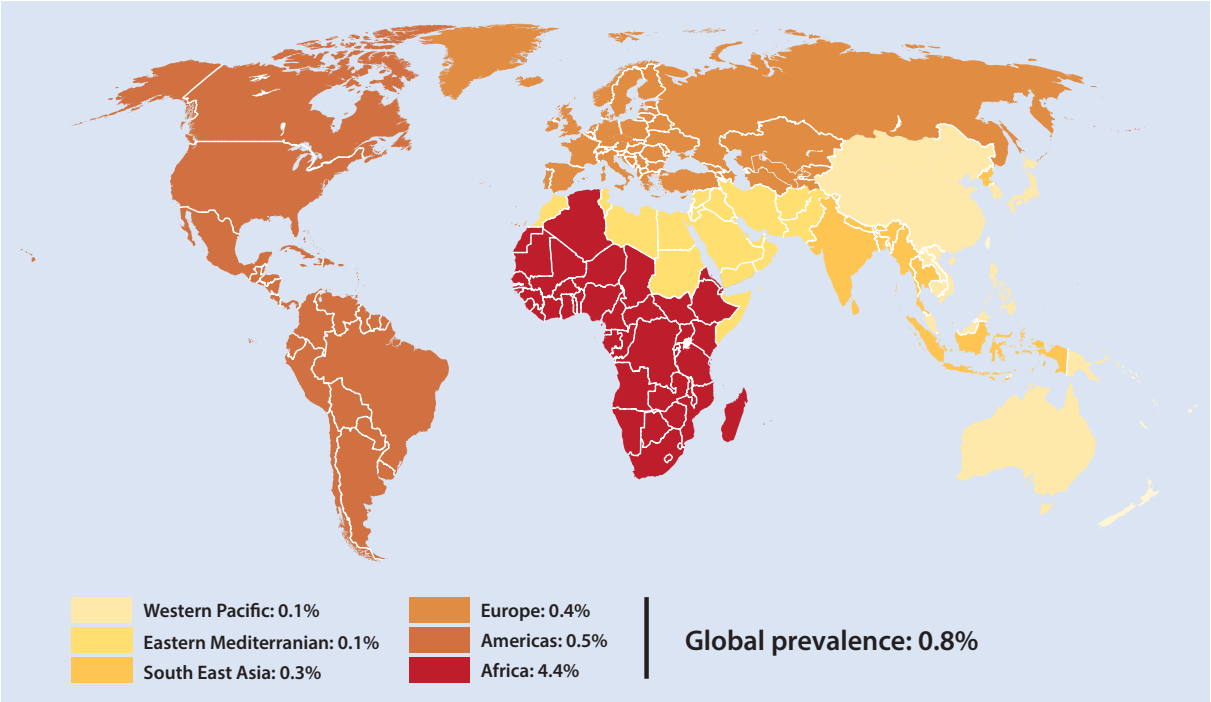

Fig. 1: Global prevalence of HIV infection in adults (15-49 years), by WHO region, in 2015. Modified from WHO.

According to Norwegian public health authorities, from the start of the epidemic through 2015, 5843 people have been diagnosed with HIV infection in Norway. In 2015, 221 new cases were reported, a reduction from 299 in the peak year 2008. Modes of transmission in these cases were 62% heterosexual, 32% homosexual, 4% by intravenous drug use and 1% vertical (mother to child) [17].

1.1.3 Transmission

Despite the scale of the global HIV pandemic and the fear it evokes, HIV is not particularly contagious compared with many other viruses. In untreated infection, the transmission probability per sexual contact has been estimated at less than 0.1% for vaginal intercourse [18]. However, this risk is substantially increased by the presence of other sexually transmitted infections, elevated viral loads in early or late-stage infection, commercial sex exposure, and in the case of receptive anal intercourse. Male circumcision has been shown to effectively reduce the risk of transmission to men [19, 20], and evidence for the efficacy of ART drugs as pre-exposure prophylaxis (PrEP) in high-risk groups is mounting [21]. In the case of ART-treated HIV infection, the risk of transmission is considered very low, but not zero [22, 23].

The risk of transmission related to intravenous drug use is hard to quantify at a per-exposure level, but has been modelled at 0.7-0.8% [24, 25]. Nonetheless, intravenous drug use is estimated to account for one tenth of new HIV infections worldwide, considerably more in parts of Eastern Europe and Central Asia [26].

The risk of transmission from mother to child during pregnancy, delivery and breast feeding is estimated at 25-40% in the absence of treatment [27]. This can be reduced significantly by ART in mothers and new-borns [28], and in countries where comprehensive prevention of mother-to-child transmission (PMTCT) programmes are available, transmission rates can be less than 0.5% [29].

1.2 Virology

1.2.1 Genetic heterogeneity

HIV is a lentivirus, part of the retrovirus family, and thus characterised by the reverse transcription of its RNA genome into DNA, which is subsequently integrated into the genome of a host cell. Due in part to an inaccurate reverse transcriptase enzyme [30] and high rate of replication in the host [31], HIV exhibits considerable genetic diversity. HIV-1 variants are classified into four major phylogenetic groups: M (main), O (outlier), N (non-M, non-O) and P. Group M viruses are responsible for the global HIV pandemic, while the other three are rare and chiefly confined to West-Central Africa [32, 33]. Group M is further divided into subtypes A-K, of which subtype C is most prevalent in sub-Saharan Africa and India and subtype B is responsible for the majority of infections in Europe and North-America. In addition, circulating recombinant forms (CRFs) exist, evidence of recombination between two viruses of different subtypes infecting one host.

1.2.2 Structure

The HIV genome is 9,7 kilobases long, and is found in two copies of positive-sense single-stranded RNA in each viral particle [34]. It consists of 9 genes: gag, pol, env, tat, rev, vif, vpr and nef, flanked by 5' and 3' long terminal repeats (LTRs).

The HIV-1 virion is roughly spherical and approximately 120 nm in diameter. The two RNA strands of the genome are accompanied in the virion core by the nucleocapsid protein p7 and viral enzymes reverse transcriptase and integrase. A cone-shaped capsid composed of p24 encloses the core. Below the lipid bilayer of the viral membrane lies the p17 matrix. Anchored in the lipid membrane are HIV envelope spikes, each a trimer of three heterodimers of gp120 and the transmembrane gp41 [35, 36].

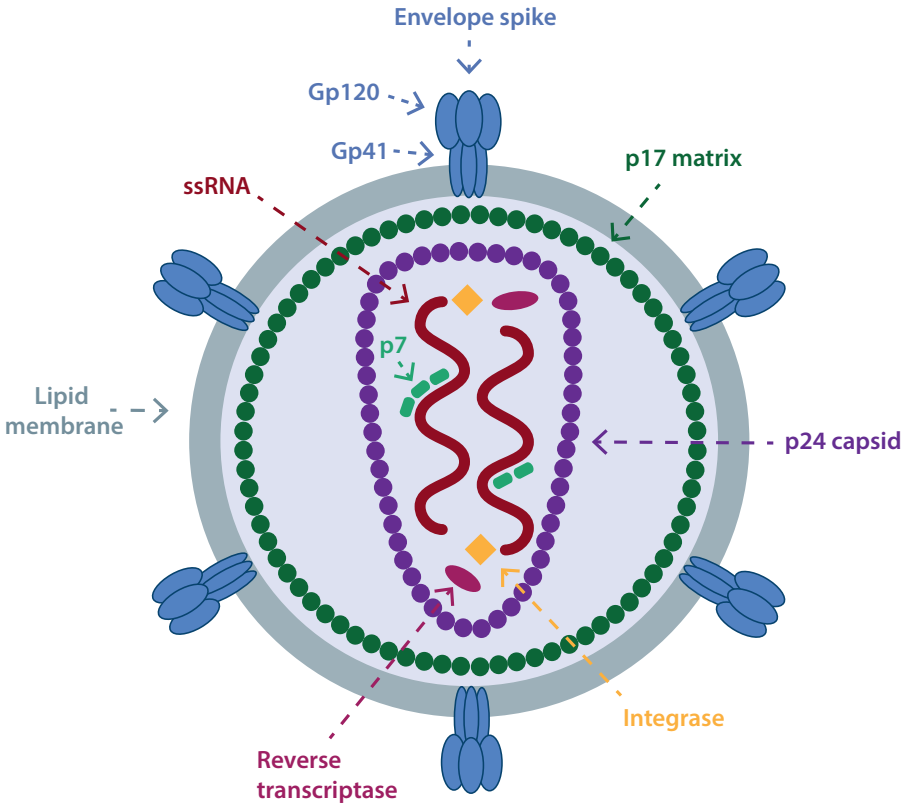

Fig. 2: Structure of the HIV virion. Modified from NIAID.

1.2.3 Replication cycle

HIV can encounter a susceptible cell by free diffusion through the extracellular fluid, but is transmitted much more efficiently by direct cell-to-cell routes, such as virological synapses, filopodial bridges and intercellular nanotubes [37-39]. In both cases, infection commences by the binding of gp120 of the envelope spike to CD4 on the target cell (Fig. 3, step 1). This induces a conformational change in gp120, allowing it to bind a second cellular receptor, typically (but not exclusively) chemokine receptors CCR5 or CXCR4 [40-43]. Further conformational changes allow gp41 to enter the cell membrane, pulling the two membranes together and initiating fusion (step 2) [44].

As the viral core is introduced into the cell cytoplasm, the capsid gradually dissolves as reverse transcriptase synthesizes double-stranded viral DNA, which along with both viral and host proteins forms a pre-integration complex (PIC, steps 3 and 4) [45]. The PIC enters the nucleus (step 5), where viral integrase cuts the chromosome and inserts the viral DNA strand, from then on referred to as the provirus (step 6) [46].

At this point the cell is irreversibly infected, and transcription of the embedded provirus may begin. Host RNA polymerase II binds to the promoter in the 5' LTR, and begins to synthesize RNAs (step 7). HIV transactivator protein Tat boosts transcription [47], and while multiply-spliced RNAs are readily exported from the nucleus, the viral protein Rev is required for the export of full-length and singly-spliced transcripts to the cytoplasm (step 8) [48]. After translation on ribosomes (step 9), the viral proteins assemble at the plasma membrane, orchestrated by the Gag polyprotein, which also recruits two copies of the viral RNA genome to be packaged into the nascent virion (step 10) [49, 50].

The virus usurps the host cell ESCRT (endosomal sorting complexes required for transport) machinery to bud off into the extracellular space (steps 11 and 12). More or less concomitantly, HIV protease cleaves the Gag and Gag-Pol precursor proteins into structural components p17 (matrix), p24 (capsid) and p7 (nucleocapsid), as well as enzymes integrase, reverse transcriptase and protease [51]. This final step in the maturation of the viral particle renders it capable of infecting a new cell (step 13).

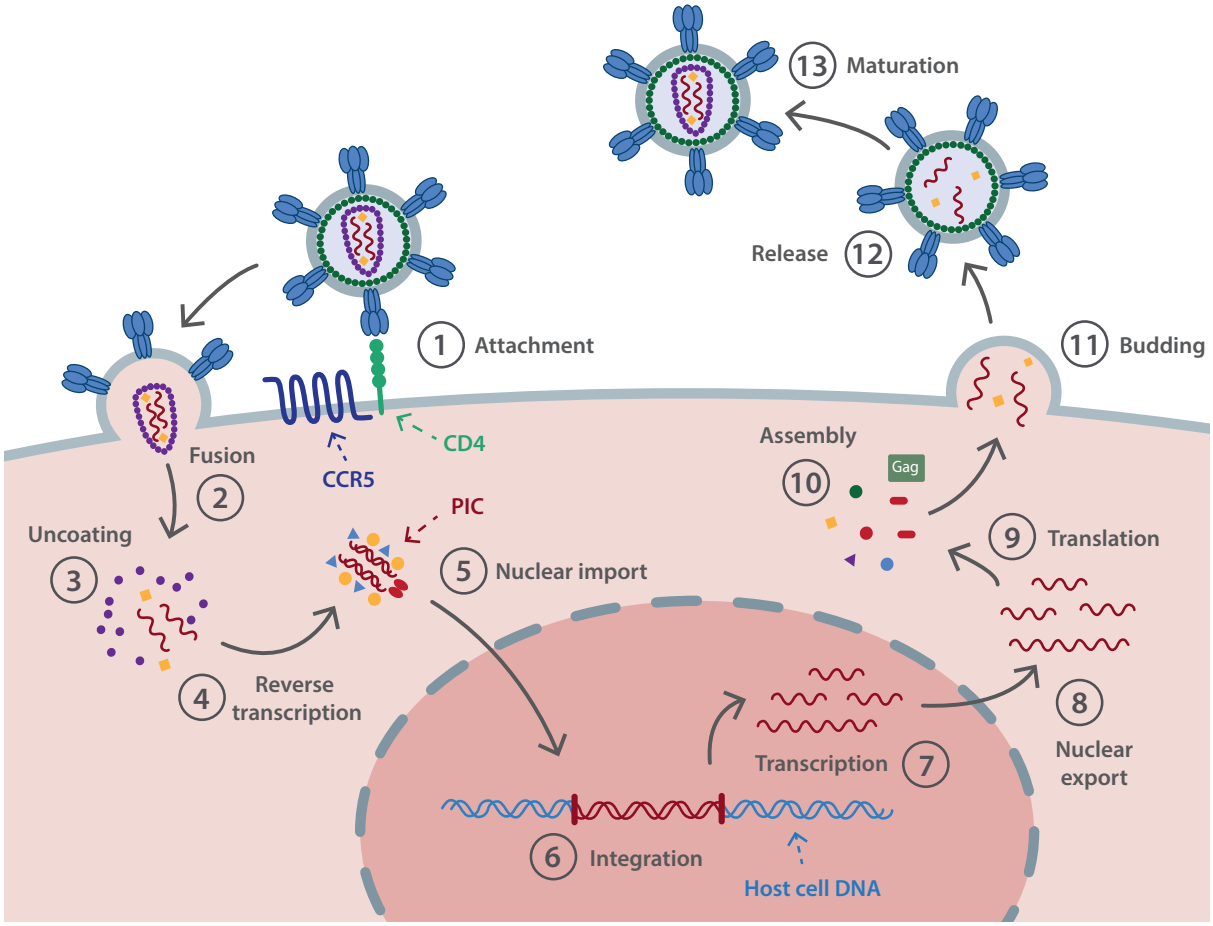

Fig. 3: Cycle of HIV replication. Modified from [52].

1.3 Clinical characteristics of HIV disease

1.3.1 Course of untreated disease

The clinical course of HIV infection has typically been divided into three phases [53]. The acute phase begins three to six weeks after transmission, when a majority of patients develop an illness characterised by fever, malaise, pharyngitis, myalgia, lymphadenopathy and maculopapular rash [54, 55]. This coincides with the peak of plasma viraemia, which can exceed 10^7 copies/mL [56], and a sharp decline in circulating $CD4^+$ T cells [55]. Symptoms may last from a few days to several months, but typically less than two weeks [57].

As symptoms resolve, viraemia rapidly decays, reaching a stable plateau known as the viral set point within three to six months [56]. This marks the beginning of a period of so-called clinical latency, in which patients tend to have mild symptoms, if any. Fatigue and lymphadenopathy are not uncommon, along with oropharyngeal and vulvovaginal candidiasis, and more frequent and severe manifestations of herpes zoster and varicella zoster infection may occur [58].

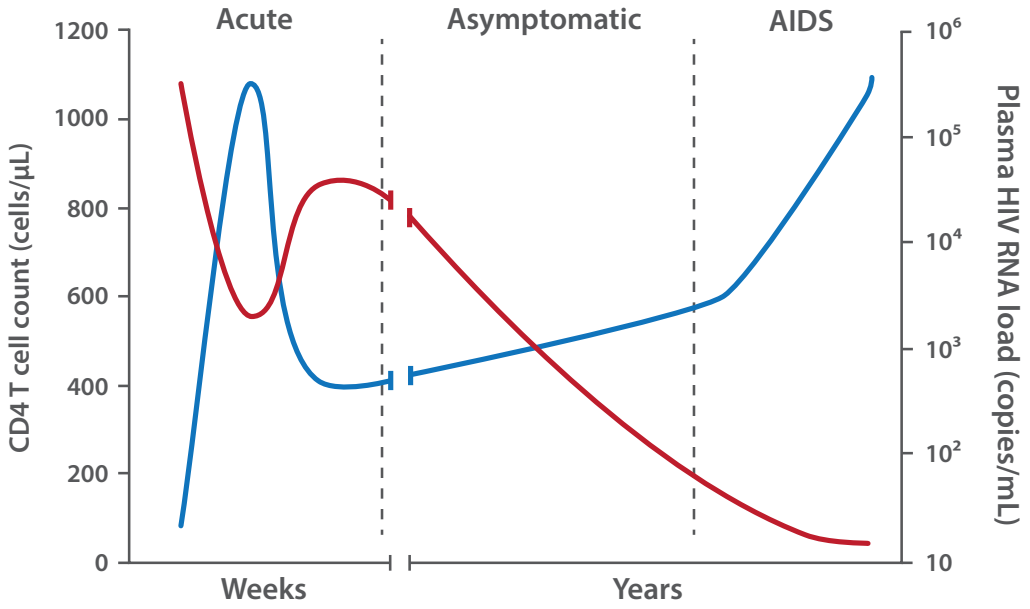

Fig. 4: Clinical course of untreated HIV infection. The first weeks of infection see an exponential increase in plasma viremia (blue line) and a massive loss of $CD4^+$ T cells, particularly in the gut, but also in the blood (red line). After acute infection, viremia drops and CD4 counts recover somewhat. In this period of "clinical latency" most patients have only modest symptoms. As the chronic phase of infection progresses, however, immune control is gradually lost, and patients reach the end-stage of HIV infection, known as AIDS. Modified from [66].

Throughout clinical latency the immune system nevertheless sustains damage, most clearly characterised by the gradual loss of $CD4^+$ T cells in the peripheral blood. Late-stage HIV infection is marked by CD4 counts below 200 cells/μL, rising viraemia and the appearance of opportunistic infections and malignancies. When AIDS develops and patients remain untreated, median survival is less than three years [59].

The rate of HIV disease progression is highly variable between individuals, and while the median time to development of AIDS is around ten years [60, 61], some patients have reached this stage within months of being infected [62]. In contrast, a small minority of so-called long-term non-progressors maintain high CD4 counts and remain AIDS-free for more than a decade of infection [63]. An even rarer patient phenotype is constituted by controllers, who are able to maintain plasma viraemia at low or undetectable levels for extended periods in the absence of ART. However, disease progression occurs in almost all patients at some point [64, 65].

1.4 Antiretroviral therapy (ART)

1.4.1 Antiretroviral drug classes

The first antiretroviral agent to see clinical use against HIV infection was reverse transcriptase inhibitor zidovudine, also known as azidothymidine or AZT [67]. Approved by the US Food and Drug Administration in 1987, it rapidly gained widespread use. However, resistance to this monotherapy was quick to develop [68], and the true revolution occurred a decade later, when regimens of three antiretrovirals in combination were shown to durably suppress viral replication [69, 70]. This so-called highly-active antiretroviral therapy (HAART, hereafter simply termed ART) has turned HIV into a manageable chronic condition in most patients with access to treatment.

1.4.2 Universal ART?

Current ART regimens suppress plasma HIV viraemia to below the limit of detection by routine clinical assays in most treated patients [72, 73]. Furthermore, patients who maintain sustained viral suppression and CD4 counts above 500 cells/μL approach the life expectancy of the general population [74, 75]. The question of when to initiate ART in HIV-infected patients has long been the subject of debate, with clinicians typically being guided by CD4 count thresholds. However, recent landmark studies have provided evidence both of a clinical benefit of early ART regardless of CD4 count [76, 77] and of a

Table of ART drugs (reviewed in: [71])

Drug class	Drug	Method of action
Nucleoside reverse transcriptase inhibitors	Zidovudine Abacavir Lamivudine Tenofovir	Incorporate into and terminate nascent DNA transcripts
Non-nucleoside reverse transcriptase inhibitors	Nevirapine Efavirenz Rilpivirine	Bind to an allosteric site on reverse transcriptase, inhibiting the viral enzyme non-competitively
Protease inhibitors	Ritonavir Atazanavir Indinavir Darunavir	Competitive blockers of HIV protease, which cleaves Gag and Gag-Pol precursor proteins as the viral particle matures
Integrase inhibitors	Raltegravir Dolutegravir Elvitegravir	Prevents HIV integrase from inserting viral DNA into the host cell chromosome
Entry inhibitors	Maraviroc	Binds to chemokine receptor CCR5, preventing its association with HIV gp120. Many but not all strains of HIV are dependent on CCR5 for cellular entry
Fusion inhibitors	Enfuvirtide	Binds to the gp41 subunit of HIV envelope, preventing fusion of the viral membrane with that of the cell

reduced incidence of new HIV infections when ART coverage is high [78, 79]. This has prompted a change in international guidelines, which now advise the consideration of all patients for ART [80, 81], and provided further impetus to expand ART access globally.

Successful treatment of HIV infection relies on a "care cascade" [82], for which UNAIDS has set ambitious future targets; in 2020, 90% of the world's HIV-infected people should be diagnosed, 90% of diagnosed patients should be on ART, and 90% of ART-treated patients should be virally suppressed. In 2030, the goal is to reach 95% in all these metrics, and thereby eventually end HIV as a public health threat [83]. However, progress towards these targets has been slow in many countries, with only an estimated 46% of all patients infected with HIV receiving ART in 2015 [15]. This percentage is the same in the hardest hit region, sub-Saharan Africa, but even lower in less impoverished parts

of the world, including Russia and the Middle East, underlining the fact that not only economic, but also political and social factors stand in the way of universal HIV therapy. Even the United States is doing poorly; of an estimated HIV-positive population of 1.2 million in 2011, only 30% were virally suppressed [84].

1.5 Immunopathogenesis of untreated HIV infection

1.5.1 Acute infection

Amplification and sequencing of viral genomes early in infection has shown that a majority of HIV infections are caused by a single founder virus [85, 86]. The virus most frequently enters the body through the genital or anorectal mucosa, where it infects intraepithelial and submucosal dendritic cells (DC) and $CD4^+$ T cells [87]. Many of these cells express CD4 and CCR5, but HIV may also use the C-type lectin DC-SIGN to enter myeloid DCs [88]. Infected DCs migrate to lymph nodes, where they encounter activated $CD4^+$ T cells which are highly permissive to infection [89, 90]. As infection is established in secondary lymphoid tissue, viral replication increases exponentially, with plasma viraemia reaching a peak after three to four weeks. Around this time, HIV-specific $CD8^+$ T cell responses can be measured [91, 92], but the fact that few viral escape mutants are present at this point shows that the immune pressure exerted by the host is entirely insufficient [85].

Plasma viraemia does subsequently fall, over a period of several months, to a so-called set point, which reflects partial immune control and is a strong predictor of subsequent disease progression [93]. $CD8^+$ T cells are thought to contribute to this partial control, as HIV-specific responses increase in tandem with the falling viraemia, and viral escape mutants rapidly appear [94].

1.5.2 Early infection in GALT

The majority of $CD4^+$ T cells in the body are found in gut-associated lymphoid tissue (GALT), where they maintain a high activation state [95], express high levels of CCR5 and are thus highly vulnerable to infection. Early HIV infection sees a massive depletion of $CD4^+$ T cells in GALT [96, 97]. This occurs both by direct cytopathic effects in productively infected cells, but to an even greater degree by bystander killing of uninfected cells by apoptosis [98, 99] and of abortively infected cells through highly inflammatory pyroptosis [100]. A subset of $CD4^+$ T cells, the interleukin (IL) 17- and 22-secreting Th17 cells, is preferentially lost [101]. These cells are important for maintaining gut epithe-

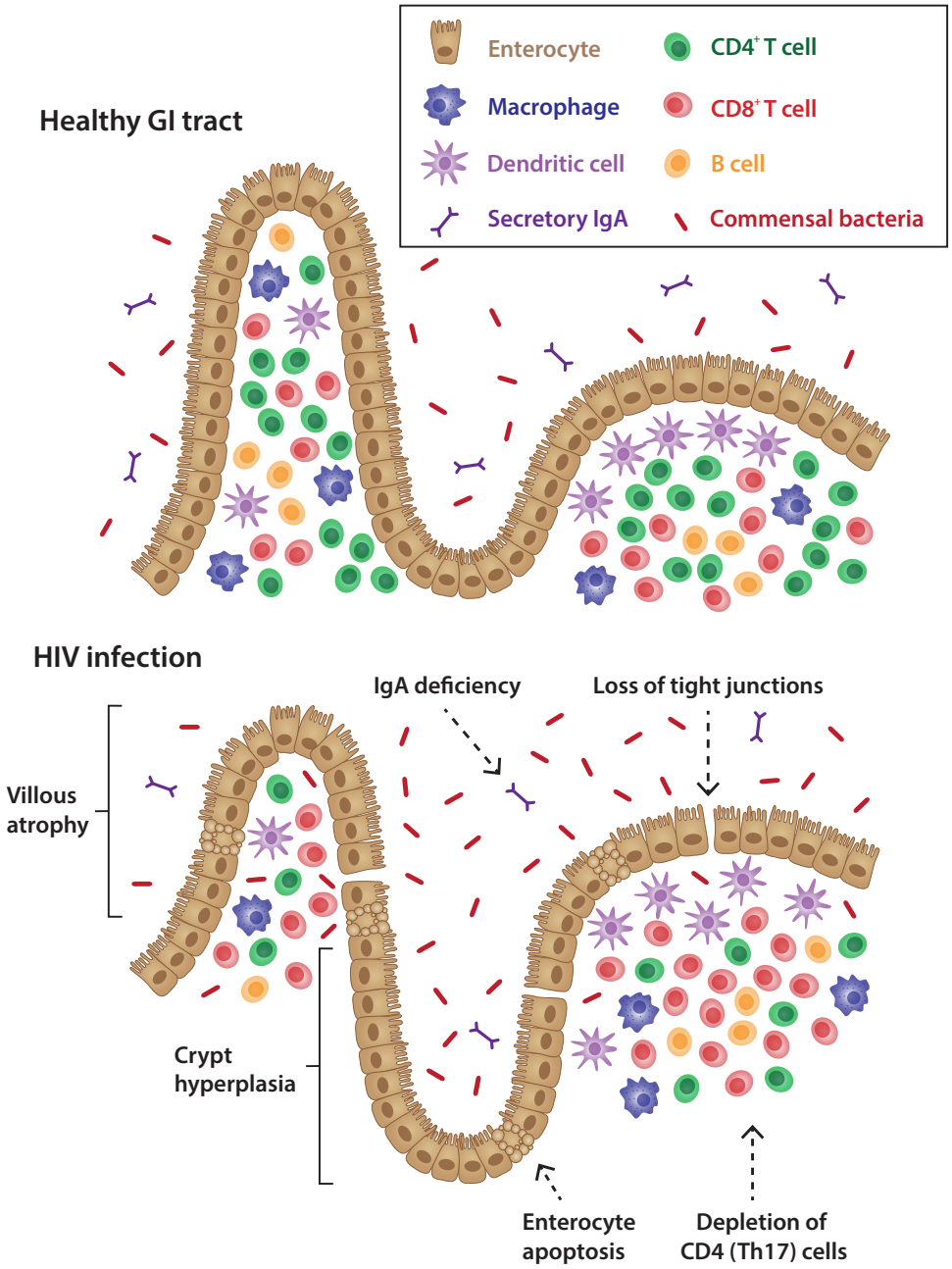

Fig. 5: Gastrointestinal pathology in HIV infection. In a healthy gut (top) the lamina propria is populated by high numbers of $CD4^+$ T cells, particularly of the Th17/22 subsets, which contribute to gut epithelial integrity. Tight junctions between enterocytes keep bacteria confined to the gut lumen. In HIV infection (bottom), the gut $CD4^+$ T cell population is massively depleted, leading to a sustained enteropathy. Microbial constituents from the gut leak into the submucosa and circulation, triggering systemic immune activation. Modified from (103).

lial integrity, and their depletion is associated with a sustained enteropathy, resulting in chronic leakage of microbial products from the intestinal lumen to the circulation, and systemic immune activation [102].

1.5.3 Chronic immune activation and inflammation

A hallmark of chronic HIV infection is a generalised hyperactivation of the immune system, as evidenced both by increased expression of activation markers on T and B cells [104, 105] and by elevated levels of circulating markers of inflammation, such as IL-1β, IL-6, tumour necrosis factor (TNF) α, soluble CD14, interferon γ-inducible protein (IP) 10 and D-dimer [106-108]. In untreated infection, plasma viraemia consistently correlates with immune activation [109, 110], and it has been shown that single-stranded HIV RNA can directly activate innate immune cells such as plasmacytoid DCs through Toll-like receptor (TLR) 7 [111]. These observations implicate viraemia itself as a primary driver of immune activation, although the aforementioned leakage of bacterial products from the gut, so-called microbial translocation, is also thought to contribute significantly. The best studied of these bacterial constituents, lipopolysaccharide (LPS) from the cell wall of gram negative bacteria, is found in elevated levels in the blood of HIV-infected patients [102] and activates monocytes and DCs through stimulation of TLR4 [112]. Activation of innate immune cells by viral RNA and bacterial products induces secretion of pro-inflammatory cytokines such as IL-6, TNF-α and type I interferons (IFN) [113]. These signalling molecules have wide-ranging effects, including the stimulation of adaptive immunity. Both $CD8^+$ and $CD4^+$ T cells of chronically HIV-infected patients, for instance, have been found to upregulate interferon-related genes, characteristic of a Th1 effector response [114, 115].

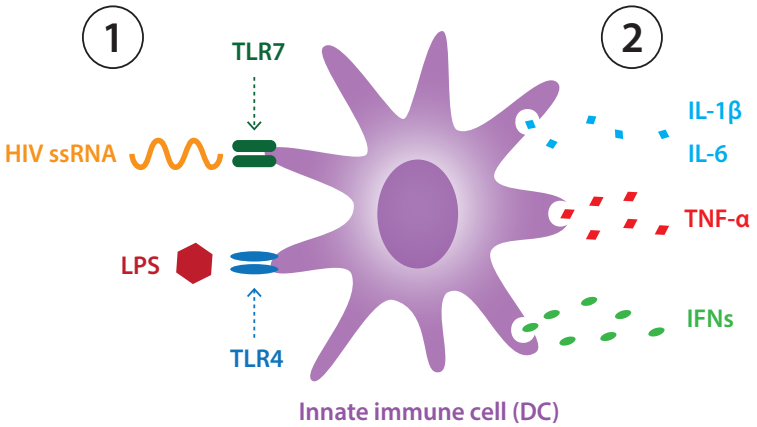

Fig. 6: Innate immune cells such as dendritic cells (DCs) and monocytes are activated via various Toll-like receptors (TLRs), both by bacterial components such as lipopolysaccharide (LPS) which have translocated from the gut, and directly by HIV RNA (step 1). This induces the secretion of large amounts of pro-inflammatory cytokines, which perpetuate the immune response (step 2).

1.5.4 Pathogenic consequences of immune activation

Observations in non-human primate (NHP) models have yielded some evidence for the pathogenic significance of immune activation. While simian immunodeficiency virus (SIV) causes a progressive, AIDS-like disease in rhesus macaques, natural hosts of SIV such as sooty mangabeys remain healthy despite long-term infection with high levels of viraemia [116]. The fact that SIV-infected macaques, like HIV-infected humans, exhibit pathological levels of immune activation, while this is lacking in mangabeys, is one of several lines of evidence implicating immune activation in HIV pathogenesis.

In untreated HIV-infected patients, immune activation, as defined by CD38 expression on T cells, and elevated plasma markers of inflammation predict disease progression and mortality [117-120], independently and better than plasma viraemia. Furthermore, immune activation is also associated with both mortality and impaired immune reconstitution in ART-treated HIV infection [121, 122], where plasma viraemia is almost completely suppressed, suggesting an independent contribution to adverse outcomes in HIV disease.

One of the mechanisms by which chronic hyperactivation compromises function in the immune system is through dysregulation of T cell homeostasis, and HIV infection leads to increased turnover of both $CD4^+$ and $CD8^+$ T cells [123, 124]. By design, a majority of T cells undergo apoptosis shortly after activation, and recent evidence suggests that abortive infection of resting $CD4^+$ T cells may contribute significantly to CD4 depletion by pyroptosis, a highly inflammatory form of programmed cell death [100]. On the supply end, inflammation-associated fibrosis and structural changes in secondary lymphoid tissue, mediated at least in part by transforming growth factor (TGF) β, is believed to compromise T cell reconstitution [125].

Another effect of chronic immune activation, along with the persistence of HIV antigens, is the development of T cell "exhaustion". This process sees T cells sequentially losing effector functions and proliferative potential, while expressing increasing numbers of inhibitory receptors on the cell surface [126].

1.5.5 Cytotoxic T cells in HIV infection

Another prime determinant of the clinical course of HIV infection is the function of HIV-specific $CD8^+$ T cells, also referred to as cytotoxic T lymphocytes (CTL). In particular, CTL responses to conserved epitopes in HIV Gag are associated with lower viral

loads both in chronic [127-129] and primary infection [130] and with greater HIV-suppressive capacity in vitro [131]. This is underscored by another profound insight gained from SIV infection of macaques, in which experimental depletion of $CD8^+$ T cells leads to rapid loss of viral control [132, 133].

So-called controllers, the small fraction of patients who maintain low or undetectable plasma viraemia in the absence of ART, have been the subject of intense study to uncover mechanisms of viral suppression. The aforementioned CTL responses against conserved Gag epitopes are more prevalent in this group [134], and many but not all have human leukocyte antigen (HLA) molecules which preferentially present such epitopes [135, 136]. In addition, controllers exhibit HIV-specific CTLs with a broader repertoire of effector mechanisms [137], implying that not only the quantity but also the quality of the HIV-specific CTL response is important.

When HIV-specific CTLs recognize their cognate antigen displayed on the HLA class I molecules of infected cells, their effector arsenal consists of several mechanisms. Firstly, they can directly induce apoptosis of target cells by the perforin/granzyme or Fas/Fas ligand (FasL) pathways. Perforin and granzyme are released into the intercellular space by degranulation, and after perforin has disrupted the target cell membrane, granzyme proteases enter the cytoplasm, initiating the caspase cascade which ends in target cell lysis [138]. Fas is a membrane "death receptor" expressed by many cells, rendering them susceptible to CTL killing. Fas is engaged by FasL on activated CTLs, also activating target cell caspases [139].

On activation, CTLs may also express a variety of cytokines, including IFN-γ, TNF-α, IL-2 and the chemokine macrophage inflammatory protein (MIP) 1β. Both IFN-γ and TNF-α increase antigen presentation on cells, and while IFN-γ renders cells more prone to apoptosis by Fas/FasL, TNF-α can induce apoptosis directly through TNF receptor I [140]. IL-2, on the other hand, is the most important growth factor for T cells, thus contributing to rapid expansion of effector cells in an ongoing immune response. As a chemokine, MIP-1β attracts leukocytes to areas of ongoing infection [141]. With its cognate receptor being the HIV co-receptor CCR5, it also has HIV-suppressive properties, at least in vitro, by blocking the receptor and promoting its internalisation [142, 143]. Furthermore, the capacity of $CD8^+$ T cells to secrete large amounts of MIP-1β on stimulation has been linked with HIV controller status and full viral suppression on ART [144, 145]. However, the in vivo significance of MIP-1β in HIV infection is still unclear, not least because T cells may not be the principal cellular source of plasma MIP-1β. In paper

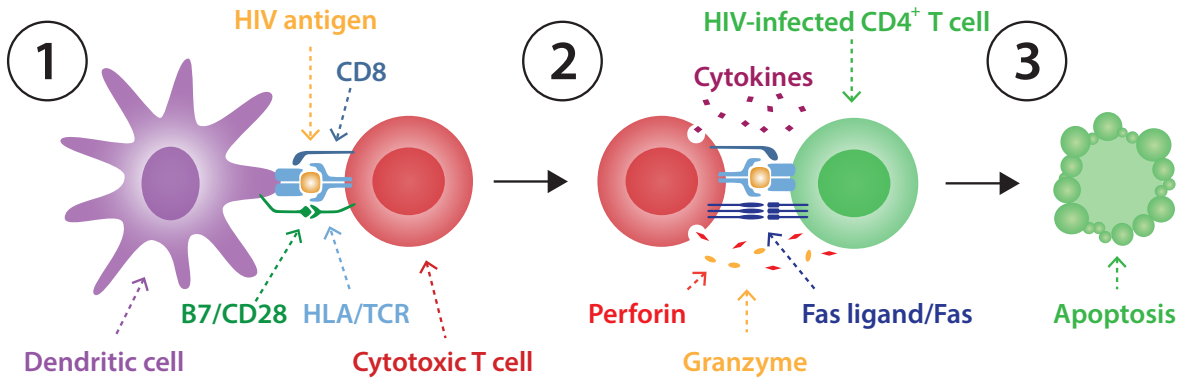

Fig. 7: Cytotoxic CD8⁺ T cells are primed by professional antigen-presenting cells (APCs) such as dendritic cells, which process and present HIV peptide antigens on HLA molecules. To be activated the T cell receptor (TCR) must match the antigen, and the T cell must be sufficiently co-stimulated via B7/CD28 interaction (step 1). When this primed T cell meets an HIV-infected CD4⁺ T cell presenting the same antigen on its surface, it is vigorously activated and deploys a variety of effector mechanisms to induce programmed cell death, or apoptosis, in the infected cell (steps 2 and 3).

I of this thesis, we identify plasma MIP-1β as a potential biomarker of immunological reconstitution on ART.

Unfortunately, HIV-specific CTL function is progressively lost during the chronic phase of HIV infection, due to a combination of immune exhaustion, clonal deletion [146] and reduced help from CD4⁺ T cells [147].

1.5.6 CD4⁺ T cell function in HIV infection

Although CD8⁺ CTLs are attributed the principal role in control of HIV infection, there is both direct and indirect evidence suggesting an essential contribution from CD4⁺ T helper cells, the virus' main cellular targets. In another NHP study, for instance, macaques depleted of CD4⁺ T cells before SIV infection had no post-peak decline in viraemia in the acute phase, and progressed rapidly thereafter [148]. Furthermore, while highly activated antigen-presenting cells (APCs) in HIV infection may obviate the need for CD4 help in the primary activation of HIV-specific CTLs [149], mouse models indicate that it is obligate for the generation and maintenance of memory CD8⁺ T cells [150, 151].

Naïve CD4⁺ T cells are typically activated by APCs in lymph nodes, and depending on the subset of APC and the cytokine milieu, activated CD4⁺ T cells undergo functional polarisation, gaining characteristic effector functions. While CD4 cells exhibit func-

tional plasticity in vivo, several functionally distinct subsets of $CD4^+$ T cell have been recognised, including the classical Th1 and Th2, the IL-17-secreting and pro-inflammatory Th17 cells, regulatory T cells (Treg) and follicular helper T cells, which assist B cell development in germinal centres [152].

HIV-specific CD4 responses are primarily Th1-polarised, characterised by production of IFN-γ, TNF-α and IL-2 [153]. In most patients, both the capacity of HIV-specific CD4+ T cells to produce effector cytokines and in particular the capacity to proliferate is compromised [154]. By contrast, controller patients maintain robust HIV-specific CD4 responses in both acute [155] and chronic infection [156, 157]. The fact that viral suppression by ART does not restore CD4 responses [153] suggests that the strong responses in controllers in untreated infection are not merely a consequence of low viraemia, but may contribute to viral control.

1.5.7 Regulation of T cell function

T cells are important not only in anti-viral, but also anti-cancer immunity [158]. However, in order to avoid excess host tissue damage and autoimmunity, their activation and function is tightly controlled. A complex system of co-signalling receptors regulates the functional nature of the activated T cell, or indeed whether the T cell becomes activated at all [159]. In order to mount a T cell response against HIV, naïve T cell clones with an HIV-specific T cell receptor (TCR) must first be primed by APCs presenting their cognate HIV antigens on MHC molecules. The archetypal co-stimulatory molecule is CD28, which interacts with B7-1/2 on APCs and without which the T cells will often become anergic or die after the APC encounter [160].

Many negative modulators of TCR signalling and T cell activity have also been identified, in the form of both membrane-bound co-inhibitory receptors, soluble regulatory molecules and even regulatory T cell subsets. The co-inhibitory receptor which has received the most attention in relation to HIV infection is programmed death (PD) 1. PD-1 gained its name when it was first identified in cells undergoing apoptosis [161], but its expression is induced in most T cells after activation, and it constitutes a physiological negative feedback mechanism to prevent excessive T cell activation [162]. However, HIV-specific T cells typically express high levels of PD-1, and PD-1 expression in HIV patients is associated with both T cell functional impairment and disease progression [163, 164]. Other co-inhibitory molecules which have been associated with functional impairment of T cells in HIV infection are cytotoxic T lymphocyte antigen (CTLA) 4 [165], lym-

phocyte activation gene (LAG) 3 [166], T cell immunoglobulin and mucin-containing protein (TIM) 3 [167], CD160 [168] and T cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT) [169].

As mentioned previously, persistent exposure to antigen and chronic activation is thought to induce an exhausted state in HIV-specific T cells, which is characterised by the progressive loss of effector functions and expression of several co-inhibitory molecules [126]. This phenomenon is also evident in other chronic viral infections and in cancer, and these insights are leading to promising new immunotherapies [170-172].

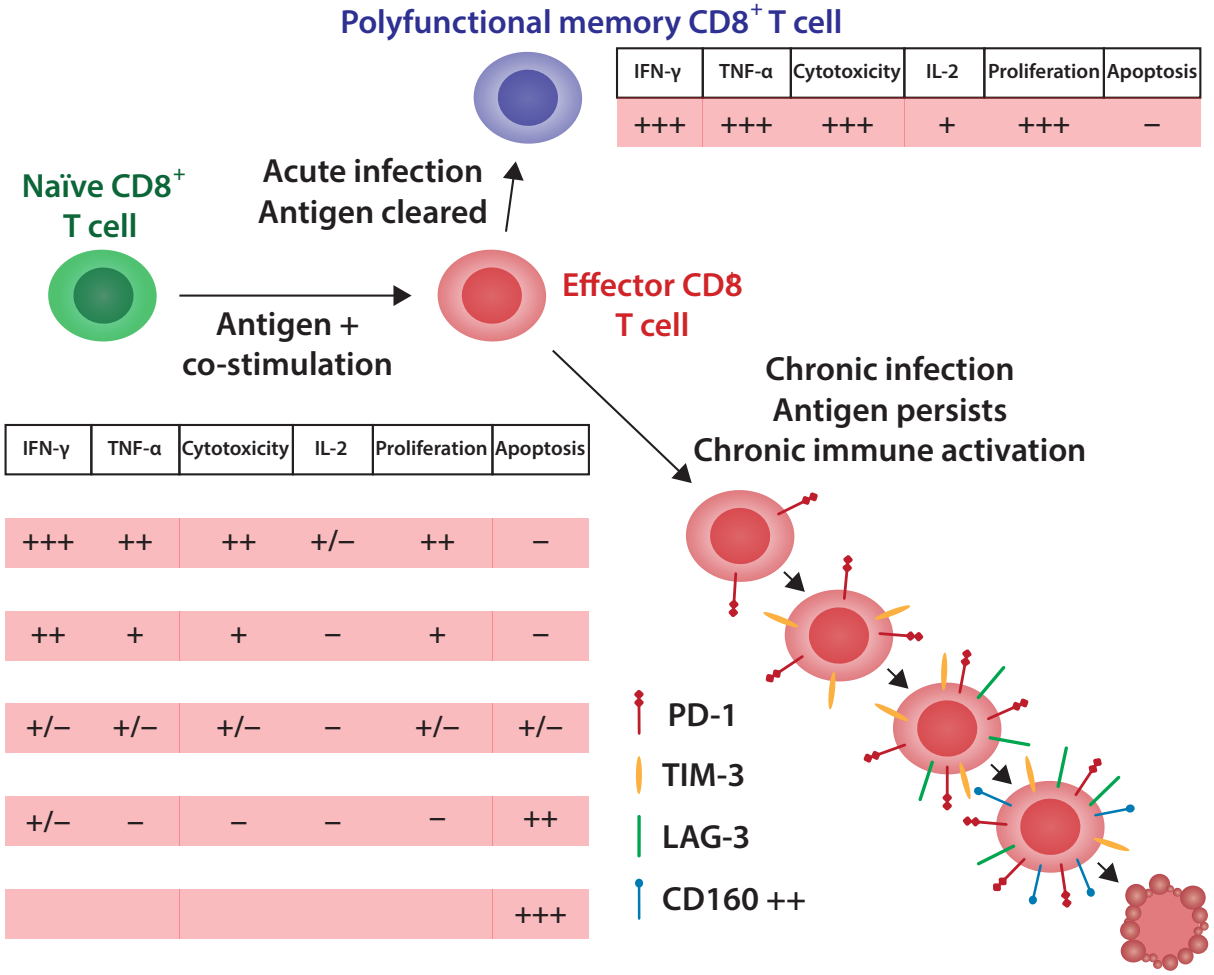

Fig. 8: In acute infections, activated CD8+ T cells differentiate into effector cells, contribute to clearing the offending pathogen, and a small proportion persist as long-lived and highly functional memory T cells, which can rapidly respond to a new challenge by the same pathogen. Chronic infections such as HIV evade immune clearance, and the persistence of HIV antigen gradually induces a state of so-called exhaustion in T cells, characterised by progressive loss of function and expression of inhibitory receptors. Modified from [126].

1.5.8 Soluble regulatory factors: IL-10 and TGF-β

Soluble signalling molecules, such as cytokines, are also intimately involved in the regulation of T cell responses in HIV. Plasma levels of IL-10 are elevated in HIV infection, and increase further with disease progression [173]. This IL-10 comes from both monocytes and multiple lymphocyte subsets, and it contributes to impairment of both proliferative capacity and cytokine secretion in HIV-specific $CD8^+$ and $CD4^+$ T cells [174]. In a mouse model of chronic viral infection, combined blockade of IL-10 and PD-1 restored virus-specific T cell function in a synergistic fashion [175].

Another cytokine which is found in higher plasma levels in HIV patients is TGF-β, which like IL-10 is even more abundant in patients with advanced disease [176]. TGF-β exerts a wide range of effects on various cells, but the overall anti-inflammatory effect is illustrated by the lethal polyfocal autoimmune disease induced in TGF-β-deficient mice [177]. In murine chronic viral infection, inhibition of TGF-β signalling improves virus-specific T cell survival and effector function [178]. In vitro, HIV antigens can induce TGF-β secretion by a subset of $CD8^+$ T cells, which in turn suppresses the HIV-specific IFN-γ response of other CTLs [179]. Finally, and likely of great importance in HIV infection: TGF-β plays a role in the induction of regulatory T cells [180, 181].

1.5.9 Regulatory T cells

Several subsets of T cells have been identified which exert a negative regulatory effect on the immune response. Of these, the most important seem to be $CD4^+$ T cells expressing the forkhead box P3 (FoxP3) transcription factor, generally termed regulatory T cells (Tregs) [182]. Tregs express CTLA-4, which out-competes the important co-stimulatory molecule CD28 on T cells for binding to its ligands CD80 and CD86 on APCs, and thus attenuates T cell activation [183]. In addition, CTLA-4 upregulates the tryptophan-catabolising enzyme indoleamine 2,3-dioxygenase (IDO) in APCs [184], which has a variety of T cell-suppressive effects. Tregs are also characterised by their high expression of the IL-2 receptor α-chain (CD25), and can thus deprive local effector T cells of IL-2, their most important growth factor [185]. Moreover, Tregs express both IL-10 and TGF-β, previously discussed as significant immunosuppressive cytokines, although the role they play in Treg-mediated suppressive function is controversial [186].

The part Tregs play in HIV pathogenesis has not been well defined. Most studies find increased Treg frequencies in HIV infection, both in blood and in lymphoid tissues,

except in controllers [187]. This could indicate a role for Tregs in harmful immune dysregulation. However, as activation of conventional effector T cells can induce many of the phenotypic characteristics used to identify Tregs, such as higher CD25 and FoxP3 expression and down-regulation of CD127 [187, 188], the higher Treg frequencies observed could at least in part be secondary to HIV-associated immune activation. Returning again to a murine model of chronic viral infection, Tregs were found to contribute to $CD8^+$ T cell exhaustion in mice chronically infected with lymhocytic choriomeningitis virus (LCMV), and Treg depletion increased the number of virus-specific CTLs [189]. Whether inhibition of Tregs could represent a therapeutic opportunity in HIV-infected patients, however, is an as yet unanswered question.

1.5.10 IDO

Indoleamine 2,3-dioxygenase (IDO), an enzyme which catabolises essential amino acid tryptophan and which can be induced in innate immune cells such as macrophages and DCs by Tregs, has gained increasing attention for its role in HIV pathogenesis in recent years. Systemic IDO activity is typically expressed as a ratio between the plasma level of tryptophan catabolite kynurenine and tryptophan itself. This so-called KT ratio is elevated in HIV-infected patients [190, 191], which is not surprising, considering that IDO is also induced in innate immune cells by IFN-γ and stimulation of TLRs [192]. Furthermore, an elevated KT ratio is characteristic of advanced HIV infection [193], and despite a reduction on ART, the KT ratio is not normalised [194], and predicts mortality even in ART-treated patients [195, 196].

A number of mechanisms by which IDO contributes to HIV-related immune dysfunction have been postulated. Firstly, local depletion of tryptophan and exposure to several tryptophan catabolites, such as picolinic acid and kynurenine, activates cellular stress-response systems in effector T cells and inhibits their proliferation [197, 198]. Secondly, activation of these same systems can induce FoxP3 expression and suppress Th17 cytokine secretion in $CD4^+$ T cells, increasing the frequency of Tregs relative to pro-inflammatory Th17 cells. This is believed to compromise the gut barrier, increasing microbial translocation and systemic inflammation [191, 199]. Finally, in concert with TGF-β, IDO can maintain a long-term tolerogenic phenotype in the IDO-expressing DCs themselves [200].

1.5.11 Prostaglandin E_2 and the potential of COX inhibitors

Yet another inducer of IDO expression in DCs is prostaglandin (PG) E_2 [201], an immunomodulatory factor synthesised from arachidonic acid by the enzymes cyclooxygenase (COX) 1 and 2 and PGE synthases. Apart from its induction of IDO, PGE_2 has been shown to attenuate T cell activation by increasing intracellular concentrations of cyclic AMP [202]. This effect is thought to be mediated by protein kinase (PK) A signalling, which is increased in the T cells of HIV-infected patients, and can be abrogated in vitro by PKA antagonists [203]. In addition, elevated cyclic AMP levels may contribute to apoptosis of CTLs via the pro-apoptotic protein Bim [204]. Recent experiments in LCMV-infected mice have shown that PGE_2, in tandem with PD-1, contributes to impaired CTL function and survival in chronic viral infection [205]. Several trials of COX inhibitor treatment in HIV-infected patients have been performed [206-209], including the study described in paper III of this thesis.

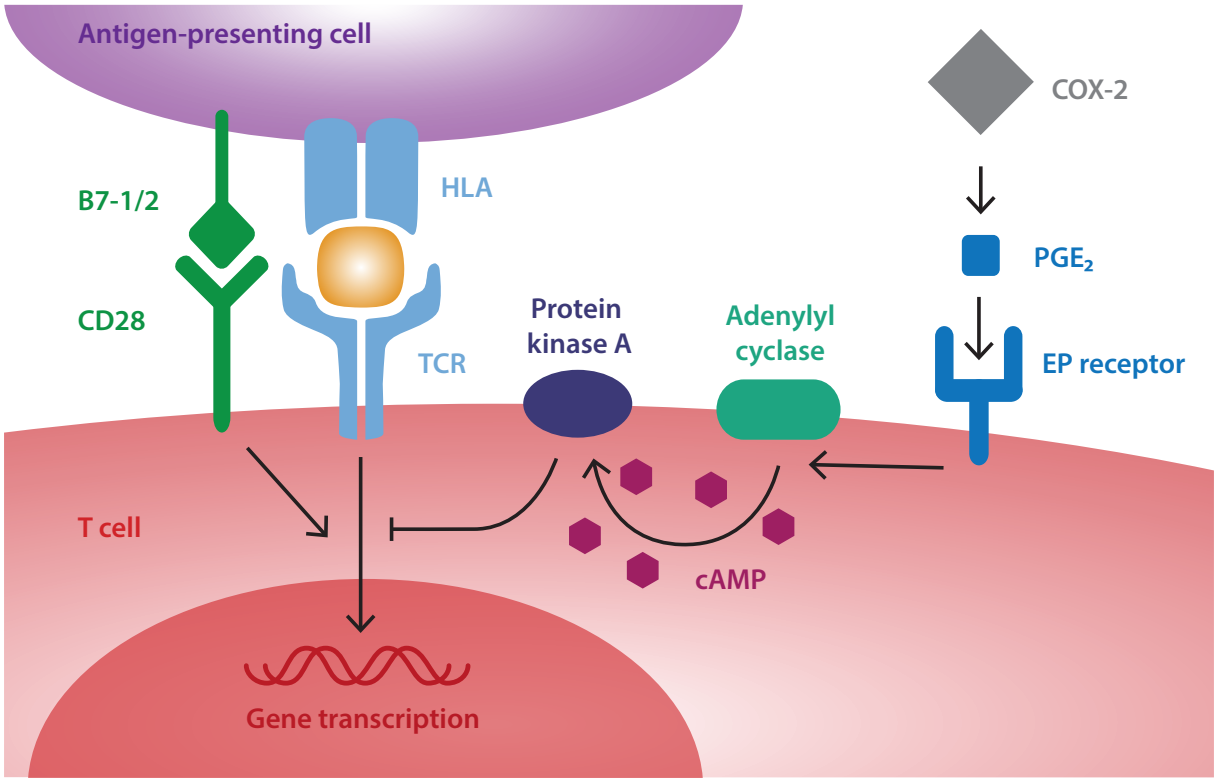

Fig. 9: Inflammation induces the enzyme cyclooxygenase (COX) 2 in innate immune cells, which leads to increased local concentrations of prostaglandin E_2. The binding of prostaglandin receptors on T cells activates adenylyl cyclase, leading to increased intracellular concentrations of cyclic AMP. This in turn leads to protein kinase A-dependent inhibition of T cell receptor signalling and reduced T cell activation.

1.6 HIV infection in the age of ART – current and future challenges

1.6.1 Morbidity and mortality in ART-treated HIV infection

While the introduction of ART represented a paradigm shift in the field of HIV care, clinical challenges remain. As people live longer with HIV, increasing evidence has demonstrated an elevated risk of typically age-related, non-AIDS conditions in ART-treated HIV infection, including cardiovascular disease [210], diabetes [211], osteoporosis [212], kidney disease [213], frailty [214] and certain forms of cancer [215]. This increased risk of non-AIDS disease has been linked to long-term toxic effects of ART drugs, a higher prevalence of traditional risk factors such as tobacco, alcohol and other substance abuse and to HIV-associated chronic inflammation [216].

1.6.2 Immunological non-response to ART

The proportion of patients who, despite sustained viral suppression on long-term ART, do not experience a normalisation of their CD4 counts, constitutes a particular clinical challenge. These so-called immunological non-responders (INR) have an increased risk of both AIDS-related and non-AIDS-related morbidity and mortality, compared to patients with adequate immune reconstitution on ART [217-220]. There is no consensus on how to define INR, hence prevalence numbers vary from approximately 15 to 30%. The phenomenon has been associated with old age, hepatitis C co-infection and most significantly, a low nadir CD4 count [221].

Despite these known risk factors, the pathogenic basis for INR development is incompletely understood, and therapeutic options to reliably boost immune reconstitution in INR are lacking. Improved means for the early identification of patients at risk of becoming INR may be useful, both for inclusion into intervention trials, and for targeted use of future adjuvant therapies to reduce the excess risk of disease in these patients. In paper I of this thesis, we present data suggesting plasma levels of the aforementioned chemokine MIP-1β as a potential biomarker predictive of INR development.

1.6.2.1 Mechanisms underlying immunological non-response

The fact that a low pre-treatment CD4 count is the strongest risk factor for INR development [222-224] suggests that the immunopathology of advanced HIV infection is less easily reversed by ART. There is evidence that INR have a leakier gut with more trans-

location of microbial products than immunological responders [225, 226], and this may in turn contribute to the higher levels of immune activation observed in these patients [227-229]. This may in turn drive a higher turnover and apoptosis of T cells [230] and lead to more fibrosis of secondary lymphoid tissue [231, 232], both likely detrimental to T cell recovery on ART. INR also seem to have a compromised thymopoiesis [221], limiting their ability to replenish the naïve T cell pool, and potentially accounting for the association between age at ART initiation and poor immune reconstitution found in some studies [224, 233].

As in untreated HIV infection, the role played by Tregs in immune reconstitution on ART is unclear. Studies have found higher frequencies and activation of Tregs in the peripheral blood of INR [229, 234], but lower numbers in tonsils [234], indicating altered Treg homeostasis and/or function, but whether this contributes to immunological non-response has yet to be elucidated.

While ART generally reduces plasma viraemia to below 50 copies/mL, ultra-sensitive assays can detect residual low-level viraemia in many treated patients [235, 236]. Both the cellular sources and pathogenic significance of this residual virus is unclear [237], but one study has suggested that residual viraemia is more frequent in INR [238], possibly contributing to increased immune activation and impaired immune reconstitution. However, ART intensification strategies with newer drugs such as CCR5 antagonist maraviroc and integrase inhibitor raltegravir neither reduce this residual viraemia nor significantly increase CD4 reconstitution in patients already suppressed to below 50 copies/mL [239-244].

1.6.3 Therapeutic approaches to immune activation and inflammation

With increasing evidence implicating chronic immune activation and inflammation in the pathogenesis of both untreated and treated HIV infection, therapeutic strategies to attenuate these pathogenic processes have attracted increased interest.

As previously discussed, microbial translocation from the gut is believed to be a significant driver of HIV-associated immune activation, particularly in ART-treated patients. While the loss of Th17 cells from GALT and compromised mucosal integrity no doubt contributes to this, the composition and metabolism of the microbial flora found in the intestinal lumen is also believed to play a role. The gut microbial flora, also known as the microbiota, is dysregulated in both untreated and ART-treated HIV-infected patients

[245, 246]. In an effort to restore the normal composition of the gut microbiota, both probiotics such as Lactobacilli, Bifidobacteriae [247] and Saccharomyces boulardii [248] and the non-absorbed antibiotic Rifaximin [249] have been investigated. While changes were noted in the gut microbiota of patients treated with Bifidobacteriae and Rifaximin, downstream effects on markers of microbial translocation and systemic immune activation have been modest or absent in all trials thus far. More conclusive evidence as to the merit of targeting the microbiota may be provided by an ongoing trial of fecal transplantation in HIV-infected patients (ClinicalTrials.gov Identifier: NCT02256592). A different approach to reducing microbial translocation has consisted of binding LPS-chylomicron complexes in the gut with the drug sevelamer, but while potentially beneficial effects were seen on blood lipids, levels of circulating LPS and monocyte activation marker sCD14 were not affected [250].

A number of the most commonly prescribed drugs in clinical practice have anti-inflammatory properties, and have been evaluated for potential benefit in HIV infection. Corticosteroids have broad anti-inflammatory and immunosuppressive effects, and in a clinical study in antiretroviral-naïve patients in Tanzania, the group which received low-dose prednisolone had higher CD4 counts and reduced immune activation [251]. In a pilot study, aspirin, a non-steroidal anti-inflammatory drug (NSAID) used by millions of patients for cardiovascular prophylaxis, reduced immune activation and platelet activation in ART-treated patients, indicating a potential double benefit in this patient group [208]. Our research group has studied another NSAID, the COX-2-selective inhibitor celecoxib, and demonstrated that this class of drug can reduce T cell activation markers in both untreated and ART-treated HIV patients [207, 209]. Paper III of this thesis follows up these studies, investigating the immunomodulatory effects of the COX-2 inhibitor etoricoxib.

Statins are widely prescribed to treat dyslipidaemia and prevent atherosclerosis, but are also increasingly recognised as anti-inflammatory agents. In the recent SATURN-HIV trial, 48 weeks of rosuvastatin reduced both activation markers CD38 and HLA-DR and exhaustion marker PD-1 on T cells, monocyte activation markers sCD14 and sCD163 in plasma, the expression of pro-coagulant tissue factor on circulating monocytes and markers of vascular inflammation [252].

1.6.4 Aiming for an HIV cure

Offering life-long ART to millions of people, along with managing the additional morbidity of an ageing HIV-positive population, will place an enormous burden on health systems in countries with a high HIV prevalence for many decades to come. Furthermore, people living with HIV are still subject to a high degree of social stigma in many societies. These are among the factors motivating HIV cure research, which is gaining increasing momentum [253].

1.6.4.1 Latent reservoir

The main barrier to achieving an HIV cure is the so-called latent HIV reservoir. In the large majority of patients, cessation of ART, even after many years of suppressive therapy, leads to a rapid rebound of plasma viraemia, typically within a few weeks. The primary source of this re-emergent virus is believed to be a pool of resting memory $CD4^+$ T cells, harbouring integrated HIV provirus which is transcriptionally silent, thus not generating any new viral antigens and rendering the cells more or less invisible to the immune system [254, 255]. Experimental SIV infection of macaques suggests that this latent viral reservoir is seeded within the first days of infection, before the emergence of quantifiable plasma viraemia [256]. In humans, the reservoir is remarkably stable, due to the long life and homeostatic proliferation of latently infected cells [257] and possibly due to some residual replication despite ART [254], which poorly penetrates several anatomical compartments [258, 259].

1.6.4.2 Potential cure scenarios

A true cure for HIV will require that the entire reservoir of replication-competent virus is purged from the body. This is believed to have been achieved in a single case, the so-called Berlin patient, who was treated for acute myeloid leukaemia with two rounds of allogeneic hematopoietic stem cell transplantation from a donor homozygous for the delta-32 deletion of the CCR5 gene. The transplanted cells were thus highly resistant to HIV infection, and along with myeloablative chemotherapy, full-body irradiation and graft-versus-host disease, seem to have purged the HIV reservoir, with no replication-competent virus found in any tissues studied, even after several years [260, 261].

However, due to the significant toxicity and high mortality rates associated with such aggressive cancer therapy, this approach is not feasible in most HIV patients. In addition, as only a single latently infected cell is believed to be sufficient to cause eventual viral

rebound after ART cessation, many experts question whether complete eradication of HIV in a large proportion of infected individuals is realistic at all [262]. Instead, durable remission of HIV viraemia in the absence of ART, or a so-called functional cure, may be a more viable option.

1.6.4.3 "Kick and kill"

Many HIV cure strategies will depend on reducing the size of the latent HIV reservoir. A "kick and kill" (also known as "shock and kill") strategy is frequently referenced, in which viral transcription is induced in latently infected cells (the "kick"), and these cells are eliminated by a host immune system boosted by immunotherapy (the "kill"). In latently infected cells, transcription of viral genes is actively suppressed [263], and overcoming this suppression has been attempted with a number of latency-reversing agents (LRAs). Early attempts involved gross activation of T cells using the cytokine IL-2 and anti-CD3 antibodies, but this approach was both associated with significant toxicity and alone failed to deplete the reservoir [264, 265]. More recently, inhibitors of the enzyme histone deacetylase, which controls chromatin remodelling, have shown some promise in forcing viral transcription without cellular activation [266, 267]. However, using these

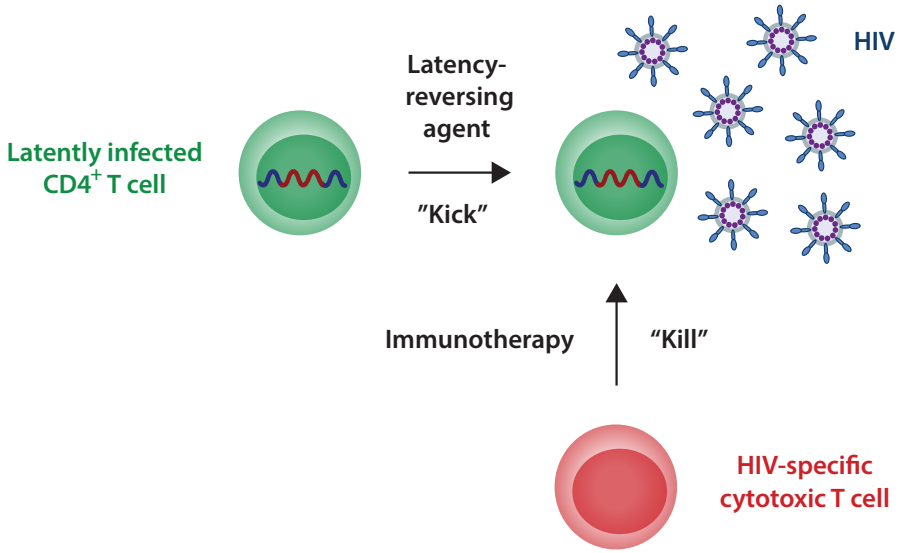

Fig. 10: The "kick and kill" approach to clearing the latent reservoir is a two-step process. First, latency-reversing agents such as histone deacetylase inhibitors and TLR agonists force transcription of HIV proviruses in latently infected cells (the "kick"), leading to cell surface display of HIV antigens and virion production. In order to ensure clearance of the reactivated cells and maintain viral control, HIV-specific immune responses, including cytotoxic T cells, are boosted by immunotherapies such as therapeutic vaccination, anti-PD-1 antibodies or broadly neutralising antibodies (the "kill").

compounds alone, reductions of the reservoir have again been minimal, and there are data suggesting that some form of immunotherapy will be required to boost killing of infected cells in this type of HIV cure strategy [268].

1.6.4.4 Immunotherapy in cure strategies

As discussed in section 1.5.5, patients with chronic HIV infection often have weak HIV-specific CTL responses, due to viral escape mutations, clonal loss, immune exhaustion and other factors. While many aspects of HIV-associated immune dysfunction is ameliorated by the initiation of ART, the HIV-specific function of $CD8^+$ and $CD4^+$ T cells is not restored [153, 269-271]. In order to enable both an initial depletion of the viral reservoir and maintain long-term viral control in the absence of ART, interventions aimed at boosting HIV-specific immunity will likely be needed. To this end, several therapeutic vaccines are being assessed, including the modified Gag peptide vaccine Vacc-4x [272, 273] and a vaccine consisting of autologous monocyte-derived dendritic cells pulsed with autologous, heat-inactivated HIV [274, 275]. While the vaccine candidates are generally safe and show in vitro immunogenicity, minimal effects on viral reservoirs and rebound kinetics have been demonstrated so far.

Another promising method of invigorating HIV-specific T cell immunity is the targeting of co-inhibitory signalling molecules, which are markers of an exhausted T cell phenotype and upregulated in HIV-specific T cells [276]. This type of immunotherapy has already been a resounding success in treating advanced cancers, where similar lymphocyte exhaustion is observed [277]. Monoclonal antibodies which block the interaction of PD-1 with its ligands, for instance, can restore CTL function in HIV-specific CTLs in vitro [278], and in vivo administration of PD-1 antibodies both improve antiviral immunity and reduce viral loads in SIV-infected non-human primates [279] and HIV-infected humanised mice [280].

There is compelling evidence that regulation of T cell responses by co-inhibitory signalling molecules has clinical significance in chronic HIV infection [145, 163], and our group has previously published data suggesting that regulatory mechanisms involving these pathways may indeed also influence the efficacy of immunotherapeutic interventions. In phase I trials of the aforementioned therapeutic vaccine Vacc-4x, regulation mediated by cytokines IL-10 and TGF-β inversely correlated with the efficacy of intradermal booster vaccination [281], whereas the presence of regulation before nasal vaccination was associated with increases in Vacc-4x-specific T cell responses [282]. This

prompted the study presented in paper II, in which a regulation assay, based on blocking inhibitory pathways, is expanded to include exhaustion markers PD-1 and CD160.

Targeting co-inhibitory molecules may not only constitute a "kill" intervention. Memory $CD4^+$ T cells which express multiple co-inhibitory signalling molecules, including PD-1, are enriched for inducible latent proviruses [283] and their frequency in the circulation predicts time to viral rebound after ART interruption [284]. Furthermore, follicular helper $CD4^+$ T cells, residing in lymph nodes and expressing PD-1, have recently been identified as an important source of inducible, replication-competent virus after long-term ART [285]. Finally, engagement of PD-1 prevents reactivation of latently infected cells on stimulation [283]. Thus, PD-1 and other co-inhibitory molecules may constitute markers of latently infected cells, and interventions to block these molecules may also have a place in the "kick" stage of cure strategies.

Finally, the observation that PGE_2 mediates suppression of CTL function in HIV infection suggests that COX-2 inhibitors may also have a role as a "kill" intervention. The hypothesis that COX-2 inhibitors can improve HIV-specific CTL function was further explored in the study described in paper III.

1.6.4.5 Early ART initiation and the latent reservoir

In vitro and non-human primate experiments suggest that the latent reservoir is established before the emergence of both plasma viraemia and clinical symptoms [256, 286], and is therefore not preventable by ART, except in the odd case of infection in spite of pre- or post-exposure prophylaxis. However, studies of patients starting ART during primary and early chronic infection have demonstrated that early therapy leads to a smaller reservoir [287-289] and lower levels of immune activation [289]. At least in theory, this may facilitate a future functional cure in these patients. Along with the established benefit of treatment as prevention [78, 79] and the improved clinical outcomes of immediate ART initiation in the START study [76, 77], this supports even more proactive HIV testing and immediate initiation of therapy in recently HIV-infected patients.

1.6.4.6 Broadly neutralising antibodies

While the importance of cellular immunity in controlling and potentially curing HIV infection can hardly be overstated, the therapeutic potential of antibodies has received increasing attention of late. This has been spurred by the isolation, after several years of infection in certain HIV-infected patients, of Env-specific antibodies which exhibit

neutralising activity against a broad range of viral strains. The structure of these broadly neutralising antibodies (bNAbs) has been resolved down to the atomic level [290], and infusion, or so-called passive immunisation, with single clones of such antibodies has been shown to reduce plasma viraemia in HIV-infected patients [291, 292]. Although resistance to this antibody monotherapy is quick to develop, this may well be overcome by cocktails of several bNAb clones, analogous to combination ART.

The most straightforward mechanism by which bNAbs exert their effect is by binding Env on free virions and preventing viral entry into cells. However, in an elegant example of humoral-cellular crosstalk, there is evidence that the Fc region mediates a significant portion of the antiviral activity of these antibodies [293, 294]. By binding both their cognate antigen at the surface of HIV-exposed cells, and Fc receptors on innate effectors such as natural killer (NK) cells, bNAbs can contribute to killing infected cells by antibody-dependent cell-mediated cytotoxicity (ADCC) [295, 296]. In combination with latency-reversing agents, bNAbs may therefore constitute yet another class of promising "kill" interventions.

1.6.4.7 Gene therapy

Considering the fact that HIV hides in our very genome, it is unsurprising that the rapid developments currently taking place in the field of genetic engineering are being applied to HIV cure research. The apparent sterilising cure achieved in the aforementioned case of the Berlin patient has largely been credited to the CCR5 deficiency of the hematopoietic stem cells he received. However, homozygosity for the delta-32 deletion in the CCR5 gene is relatively rare, and thus finding a suitable HLA-matched donor for a multitude of HIV-infected patients is not feasible. To solve this problem, a number of gene-editing tools are being used to engineer autologous, CCR5-deficient T cells ex vivo, including zinc-finger nucleases (ZFNs), CRISPR-Cas9 [297], and transcription activator-like effector nucleases (TALENs) [298]. One obvious caveat to this approach is the existence of CXCR4-tropic HIV, which can potentially infect these engineered cells.

Conceptually, an even more satisfying application of genetic engineering is the direct excision of HIV proviruses from latently infected cells. ZFNs [299] and in particular the CRISPR-Cas9 system [300, 301] are being extensively tested to this end, with promising results in in vitro studies. However, efficiently delivering these enzymes in vivo and avoiding harmful off-target effects are challenges still to overcome. Furthermore, all genetic engineering approaches to an HIV cure are resource-intensive, and serious questions have been raised concerning the scalability of their clinical application.

1.6.4.8 Ethical considerations in cure trials

A cure for HIV will, given that it is made available in the resource-limited settings where HIV is most prevalent, potentially change the lives of tens of millions of people. However, in this worthy pursuit, investigators face a number of ethical challenges, particularly when bringing interventions with promising results in the laboratory into clinical trials.

In the current early phase of cure research, most clinical trials will be proof-of-concept studies, in which study participants cannot expect any durable clinical benefits from experimental interventions, but still risk harmful side-effects and complications. Participants, who can often overestimate the benefits and underestimate the risks of early clinical trials in general [302], must be well informed of this for their recruitment to be ethical and their consent to be valid.

Furthermore, while certain oncology trials can reasonably recruit seriously ill patients with less to lose for risky interventions, HIV-infected patients on ART generally have an excellent prognosis and minimal symptoms, allowing a considerably lower level of acceptable risk in HIV cure trials. As the real test of any cure strategy is the time to viral rebound after ART cessation, many cure trials will need to include analytic treatment interruptions (ATIs), or as they have recently been repackaged; intensively monitory antiretroviral pauses (iMAPs) [303]. The inferiority of intermittent vs. continuous ART was unequivocally established by the SMART trial [304], and while the risk of temporary ART interruptions should be minimised by vigilant monitoring, viral rebound may entail a risk of reseeding of the reservoir, HIV transmission, collateral damage of systemic inflammation and development of resistance to ART drugs, at least in theory. These risks must be carefully considered and communicated to potential study participants.

2. Hypotheses and summary of papers

The current paradigm defines HIV infection largely as a condition of systemic immune hyperactivation and inflammation. These processes seem to play an important role in driving immunodeficiency in untreated HIV disease as well as in many of the clinical complications of ART-treated infection, including immunological non-response and increased non-AIDS morbidity and mortality. While regulation of immune responses is vital to avoid widespread immunopathology, the induction of these regulatory mechanisms by HIV-associated immune activation and inflammation significantly impairs control of the infection. These observations have motivated the work presented in this thesis. We initiated this work with the following hypotheses:

1. *Immunological non-response to ART can be predicted by plasma markers of inflammation measured before ART initiation.* HIV-associated inflammation may play a role in poor CD4 count recovery despite effective suppression of plasma viraemia on ART.

2. *Functional regulation of HIV-specific T cell responses can be quantified in an in vitro assay.* Chronic immune activation compromises HIV-specific T cell function, which has implications both for control of viraemia in untreated infection and for immunotherapeutic interventions, including cure strategies. Quantifying this regulation may have the potential to guide immunotherapy in individual patients.

3. *A cyclooxygenase (COX) 2 inhibitor may reverse prostaglandin E_2-dependent inhibition of T cell functions and reduce generalised hyperactivation of T cells in HIV-infected patients both off and on ART.* The enzyme COX-2 is induced in innate immune cells in HIV infection, leading to increased synthesis of PGE_2, which can inhibit T cell activation and function via a cAMP/protein kinase A-dependent signalling pathway.

Three separate studies were performed to investigate these hypotheses, and the results are briefly summarised here.

2.1 Paper I

Immunological non-responders (INR) to ART are at increased risk of both AIDS-related and non-AIDS-related morbidity and mortality. To identify potential biomarkers to predict INR development and better understand the underlying pathogenesis, we performed serial analysis of 32 cytokines, chemokines and growth factors in plasma samples from 112 HIV-infected patients initiating ART. Samples were drawn pre-ART and during the first three years of treatment, and analysed by ELISA and multiplex immunoassay. Immunological response was defined as a CD4 count above 350 cells/μL, and evaluated after up to 13 years of suppressive ART.

As expected, baseline CD4 count was the strongest predictor of subsequent long-term INR in the cohort as a whole. However, in patients starting ART with a CD4 count below 200 cells/μL, baseline plasma levels of the β-chemokine MIP-1β independently predicted long-term INR, and MIP-1β remained elevated in plasma in these patients for the first three years of ART.

2.2 Paper II

HIV-specific cytotoxic T lymphocyte (CTL) function is progressively impaired in chronic HIV infection, and not restored by ART. In previous studies we have quantified the negative regulation of HIV-specific CTL function by the regulatory cytokines IL-10 and TGF-β, observing an association between regulation and both clinical progression and impaired efficacy of a therapeutic Gag-based vaccine. In this study, we expanded the same assay to include co-inhibitory receptors PD-1 and CD160, defining a parameter we term functional T cell regulation (FTR).

Twenty-six ART-naïve patients were included, and FTR associated with HIV Gag and/or Env peptide stimulation was detected in 54% of patients, exhibiting considerable heterogeneity between patients and inhibitory pathways. However, blockade of IL-10/PD-L1 and IL-10/TGF-β detected all cases of Gag- and Env-associated FTR, respectively. In accordance with previous findings, isolated Env FTR was associated with higher plasma HIV RNA and lower CD4 counts, while patients with both Gag and Env FTR also had higher Gag- and Env-induced CTL responses. While the frequency of activated regulatory T cells (Tregs) increased after both Gag and Env stimulation, there was no association between Treg frequencies and FTR.

2.3 Paper III

Pathological immune activation and inflammation contributes to HIV disease progression and immunodeficiency, and is believed to play a role in immunological non-response and non-AIDS morbidity during antiretroviral therapy. In addition, elevated levels of prostaglandin E_2 in HIV infection may attenuate the HIV-specific T cell immunity vital for most HIV cure strategies. We have previously shown that a high dose of the cyclooxygenase-2 inhibitor celecoxib can reduce HIV-associated immune activation and improve IgG responses to T cell-dependent vaccines in untreated patients.

In this follow-up study, 28 ART-naïve and 28 ART-treated patients were randomised to receive 90 mg qd of the cyclooxygenase inhibitor etoricoxib for six months, two weeks or to a control arm, respectively. In ART-naïve patients, etoricoxib reduced the density of activation marker CD38 in multiple $CD8^+$ T cell subsets, improved Gag-specific T cell responses, and reduced in vitro plasma thrombin generation, while no effects were seen on plasma markers of inflammation or tryptophan metabolism. No significant immunological effects of etoricoxib were observed in ART-treated patients. There was no beneficial effect of etoricoxib on the IgG response to three T cell-dependent vaccines.

3. Methodological considerations

3.1 Study design and participants

	Study participants	Study details
Paper I	112 patients initiating ART	Retrospective study analysing cytokines, chemokines and growth factors in biobanked serial plasma samples drawn before and during the first three years of ART
Paper II	26 ART-naïve	Quantification of HIV antigen-associated regulation of CTL responses in biobanked PBMC samples
Paper III	28 ART-naïve, 28 ART-treated	Clinical study to explore the immunomodulatory effects of COX-2 inhibitor etoricoxib

All study subjects were HIV-infected adults, recruited from the outpatient clinic of the Department of Infectious Diseases at Oslo University Hospital. All participants gave written, informed consent to participate, and all studies were approved by the Norwegian Regional Committee for Medical and Health Research Ethics South East.

Patients in the clinical study in paper III were closely monitored with regards to adverse events and safety parameters throughout the study, and the study was approved by the Norwegian Medicines Agency.

3.2 Sample processing and storage

3.2.1 Plasma and serum samples

In all three studies, plasma and serum samples were cryopreserved in a biobank and then analysed in batches. This approach aims to reduce inter-assay variability and ease study logistics, but in order for studies based on biobanked samples to yield meaningful information, it is imperative that the analytes of interest are relatively stable when cryopreserved over time. Plasma and serum is typically stored at -70°C/-80°C, and studies have shown that most cytokines, antibodies and coagulation factors are stable for several years under these conditions, provided they are not exposed to repeated cycles of freezing and thawing [305-307].

Importantly, and as discussed in paper I, samples from the main cohort analysed in that study had been stored at -20°C for up to a decade. By analysing samples from a second HIV-positive, ART-naïve cohort, and from HIV-negative controls, which had all been stored at -70°C, we identified a number of cytokines, chemokines and growth factors which were subject to significant degradation during prolonged storage at -20°C, and which were thus excluded from further analysis.

3.2.2 Cryopreserved cellular samples

All T cell phenotyping and functional assays were performed using cryopreserved peripheral blood mononuclear cells (PBMC), isolated from whole blood using BD Vacutainer Cell Preparation Tubes. These tubes use the principle of Ficoll-Hypaque density gradient separation to isolate lymphocytes and monocytes/dendritic cells from granulocytes and erythrocytes [308]. PBMC are conventionally used in functional T cell assays, as this avoids the influence of other blood components, such as granulocytes and plasma. Whole blood assays may have their advantages, more closely reflecting in vivo conditions and requiring smaller sample volumes from patients [309, 310]. However, while cryopreserved whole blood has been used in some functional lymphocyte assays [311], the use of PBMC in these types of studies is far more established. Despite the fact that long-term cryopreservation of cells may affect the results of both phenotypic and functional analyses [312, 313], consistent results can be achieved when cells are correctly processed [314, 315]. In the studies presented in this thesis, the benefits of using cryopreserved cells were considered to outweigh the disadvantages, and all T cell studies were performed in batches using PBMC which had been stored at -150°C.

All cryopreserved samples used in functional experiments were counted and checked for viability after thawing and overnight rest, by DNA-binding dye exclusion, on a Muse Cell Analyzer tabletop flow cytometer (Merck Millipore, MA, USA). Viability exceeded 70% in all samples, a threshold validated in previous studies with cryopreserved PBMC from HIV-infected patients [315].

3.3 Analysis of circulating markers

3.3.1 ELISA

In papers I and III, enzyme-linked immunosorbent assay (ELISA) was used to quantify cytokines, antibodies and other circulating proteins in plasma and serum samples.

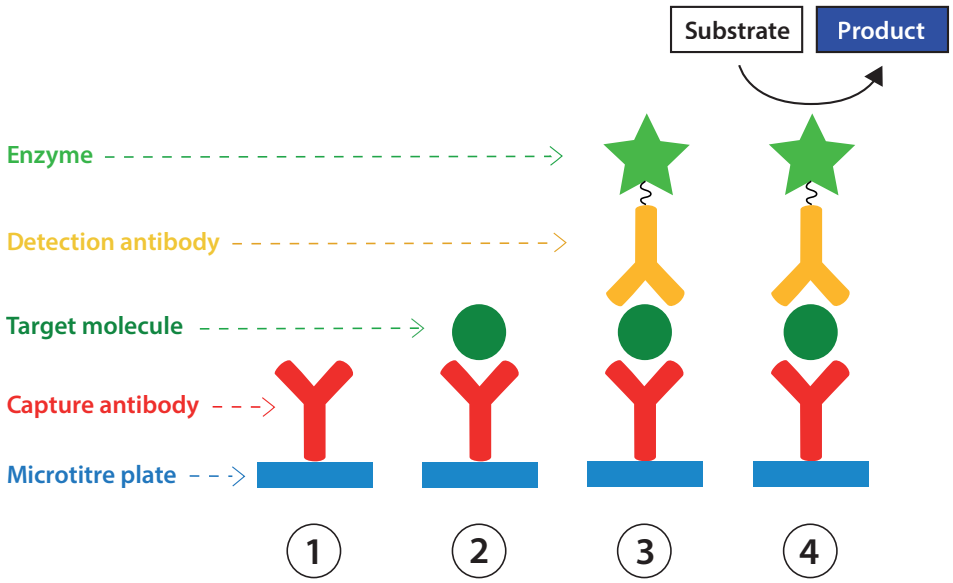

Fig. 11: Enzyme-linked immunosorbent assay (ELISA) was used to quantify cytokines, antibodies and other proteins in serum and plasma samples. Modified from [318].

While several formats exist, "sandwich" ELISA is typically performed in micro-titer plates coated with a "capture" antibody specific for the analyte of interest. To prevent non-specific binding of analyte in the well, a non-reacting protein solution is added, coating the remaining well wall. The sample is then added, and any analyte of interest present in the sample is bound to capture antibody. The well is washed to remove unbound analyte, before a solution containing more specific antibody is added, "sandwiching" analyte molecules between two antibodies. This primary antibody (direct method), or a secondary antibody specific for the primary antibody (indirect method), is linked to an enzyme, which catalyses a chromogenic reaction. The concentration of analyte in the original sample is determined by comparing light absorbance in the assay well with a standard curve generated by running several known concentrations of the analyte [316, 317].

The ELISA analyses in papers I and III were performed by collaborators at The University of Oslo, Oslo University Hospital and The Norwegian Institute of Public Health.

3.3.2 Multiplex immunoassay

ELISA, while being a convenient and sensitive method, is limited by the need for one sample aliquot per analyte to be measured. This reduces the number of molecules which can be quantified in clinical studies with limited sample volumes, and performing many separate ELISAs can be time-consuming. A more recent adaptation of enzyme immunoassays overcomes this limitation. Bead-based multiplex assays utilise micro-beads, which

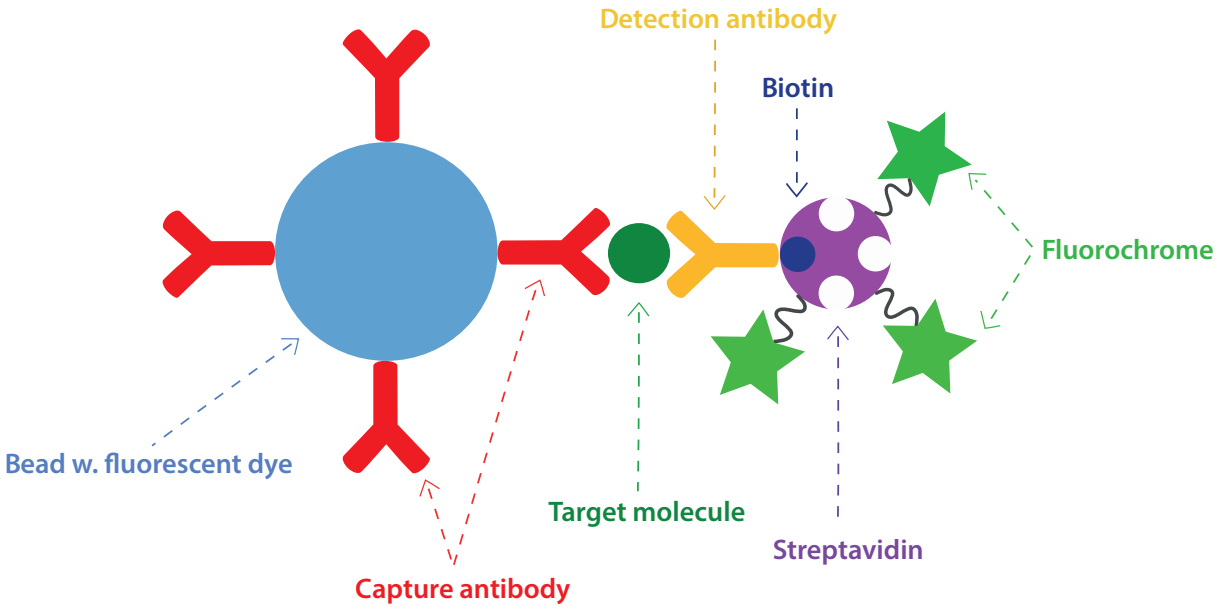

Fig. 12: Bead-based multiplex immunoassay. The capture antibody is bound to microbeads with a given fluorescent signature. A broad panel of different capture beads can be added to the same sample, allowing the quantification of numerous analytes simultaneously. Modified from [318].

are both labelled with fluorescent dyes and coated with antigen-specific capture antibodies. Beads with a large number of different specificities may be added to a single sample, along with biotinylated, antigen-specific detection antibodies and a streptavidin-conjugated reporter fluorochrome. The sample is then run on a flow cytometer, where the specificity of each bead is determined by its own fluorescent signature, while the amount of bound analyte is quantified by the fluorescence intensity of the reporter fluorochrome [319, 320]. Bead-based multiplex assays currently allow the quantitation of hundreds of targets in a single sample, with a broad dynamic range.

A bead-based multiplex assay was used in paper I to quantify the levels of 27 cytokines, chemokines and growth factors in cryopreserved plasma samples. The analysis was performed by collaborators at The Norwegian University of Science and Technology and The Nordland Hospital.

3.3.3 Liquid chromatography - tandem mass spectrometry

While immunoassays such as ELISA and bead-based multiplex assays are probably the most commonly used methods of quantifying biological molecules in plasma, the use of monoclonal antibodies for analyte detection limits their sensitivity and specificity, due

at least in part to unspecific binding. An alternative method, liquid chromatography - tandem mass spectrometry (LC-MS), can quantify molecules at lower concentrations and with higher specificity, using an entirely different principle of detection.

In LC-MS the various constituents of the sample are first separated by liquid chromatography, where the sample is pumped at high pressure past a solid adsorbent material, which differentially interacts with various molecules. Then, the sample is vaporised, ionised and the resulting ions enter a chamber of high vacuum, where they are subjected to an electromagnetic field. The mass-to-charge characteristics of the ions are detected, yielding a mass spectrum for the constituents of the sample, which can then be identified [321].

In paper III, LC-MS was used to quantify tryptophan metabolites in plasma samples. The analysis was performed on a commercial basis by Bevital AS in Bergen.

3.3.4 Calibrated automated thrombogram (CAT)

CAT is a fluorimetric method of assessing thrombin generation in plasma or whole blood, and can be used to evaluate states of both hyper- and hypocoagulability [322]. HIV infection is associated with increased systemic inflammation and levels of D-dimers [216], and the use of cyclooxygenase inhibitors affect both platelets and endothelium [323]. Thus, it was considered relevant to assess coagulation in the clinical study described in paper III, which included HIV-infected patients both on and off ART receiving Cox-2 inhibitors.

The CAT analyses in paper III were performed by collaborators at The University of Oslo and Oslo University Hospital.

3.4 Cellular analyses

3.4.1 Flow cytometry

The ability to characterize large numbers of cells on a single-cell basis has made the technique of flow cytometry a key tool in many fields of biomedical research as well as in clinical laboratory medicine. This is particularly true in immunology, where cell phenotypes, effector functions and signalling pathways have been extensively explored by this method.

Flow cytometry is based on the hydrodynamic focusing of cells in solution, sending them past an array of lasers in single file. The cells are stained in advance with fluorochrome-conjugated monoclonal antibodies, which may be specific for a wide variety of phenotypic markers, intracellular cytokines and even phosphoproteins. As the laser hits each cell in succession, light is scattered by the diffractive properties of the cell, giving information about its physical attributes (so-called forward and side scatter). Any fluorochromes present on or in the cell are excited by the lasers, emitting light at certain wavelengths. This light is directed, by way of mirrors and filters, into one of several photo-multiplier tubes (PMT), which translates it into an electrical signal and amplifies it. Through digital processing, multiple parameters for each cell are recorded for subsequent analysis.

To yield valid data, flow cytometry experiments depend on a variety of controls [324]. Firstly, the instrument must be correctly set up, the lasers precisely aligned and PMT amplification voltages suitably set. This is often controlled with standardised test beads supplied by the instrument manufacturer. All flow cytometric analyses in the work presented here were performed on a BD FACS Canto II 9-channel cytometer (BD Biosciences), and instrument set-up was controlled daily by running BD Cytometer Setup and Tracking beads.

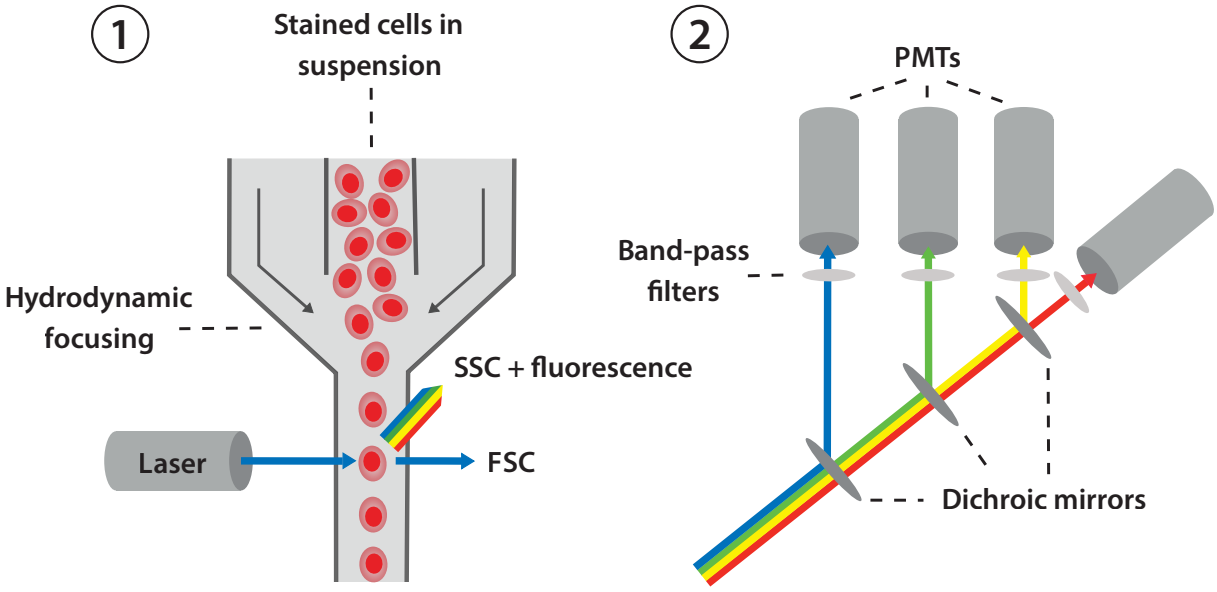

Fig. 13: Principles of flow cytometry. Fluorochrome-stained cells pass in single file past one or more lasers in the flow cell (1). Light emitted by the fluorochromes and laser-derived light scattered by the cell itself is directed into photomultiplier tubes (PMTs) via an array of mirrors and optical filter (2).

Secondly, the spillover of light from one channel to another must be determined and compensated for. The often broad and overlapping emission spectra of many fluorochromes lead to some light entering other channels than intended, giving a "false" signal in those channels. To correct for this, the process of "compensation" involves running test samples stained with each single fluorochrome in sequence, registering the spillover into other channels. This can then be automatically substracted from the signals collected when the study sample, stained with the complete fluorochrome panel, is run. We performed compensation in setting up all the flow experiments in this work, using the automatic compensation calculation of the cytometer software (BD FACS Diva). In the case of antibody-fluorochrome conjugates, compensation beads were used, whereas PBMC were used in calculating compensation for cellular dyes.

The analysis of flow cytometry data is based on "gating," that is, defining subsets of cells based on their fluorescence characteristics. When cells are unequivocally positive or negative for a marker, gates can be set by visually examining the data in histograms or bi-parametric "dot plots." However, if there is a more continuous expression of a marker, gating controls should be used. Historically, isotype controls have seen frequent use, particularly to assess unspecific staining, but also to set gates. These antibodies have the same isotype as the test antibody, and the same conjugated fluorochrome, but lack the marker-specific Fab-domains. However, there is increasing consensus that isotype controls are unsuitable to set gates, in part because fluorescence spillover from other channels tends to be a greater source of "false signal" in polychromatic flow cytometry than unspecific staining. This can be determined and corrected for by the fluorescence-minus-one (FMO) method, in which one stains control cells with all antibodies in the panel except the one for which the gate is being set. The difference between the test sample and the FMO control must be due to staining by the antibody of interest. A third type of gating control is a biological control, often used in functional experiments. In stimulation assays, for instance, unstimulated cells will often provide the best basis to set the boundary between positive and negative events [324]. Another iteration of a biological comparison control is using a similar cell population where the expression of a marker is known in advance and clearly positive or negative.

In papers II and III, FMO controls were run on all non-discrete markers in preliminary experiments. In most cases, there was no appreciable difference between FMO and completely unstained samples, and in these cases, FMO controls were omitted from subsequent runs. Where appropriate, biological comparison samples were used to set gates

3.4.2 Proliferation and regulation assays

The rationale for using peptide-stimulated proliferative responses to assess T cell regulation is discussed in paper II, and we believe it is a sound and clinically relevant approach. Proliferation was assessed by pulse-labelling cells with carboxyfluorescein diacetate succinimidyl ester (CFSE), prior to peptide stimulation and culture. CFSE is a fluorescent dye which covalently binds to intracellular molecules, where it is stable for extended periods of time, and is equally distributed between daughter cells on cell division. Thus, proliferation of cells can be monitored by flow cytometry, with CFSE fluorescence halved for each subsequent generation [325]. As CFSE has the potential to inhibit T cell proliferation at high concentrations [326], preliminary experiments were performed to ascertain the ideal concentration for labelling, 2 μM in this study.

Worth noting, however, is that not all the proliferated $CD8^+$ T cells we detect by flow cytometry after five days of culture are necessarily HIV-specific effector cells. In this type of assay, substantial IL-2 production by the antigen-specific T cells induces a certain magnitude of bystander proliferation in other cells specific for completely unrelated antigens [327]. One way of focussing our analysis on only HIV-specific $CD8^+$ T cells would be the use of fluorochrome-conjugated, soluble class I HLA tetramers [328], which bind the TCR of a single $CD8^+$ T cell clone. However, this would necessitate HLA-haplotyping of all our patients, which has yet to be done consistently in our lab. Furthermore, given the immunological and virological heterogeneity of HIV infection, using a single or a handful of clones to represent the HIV-specific $CD8^+$ T cell response in all our patients may not necessarily represent a gold standard.

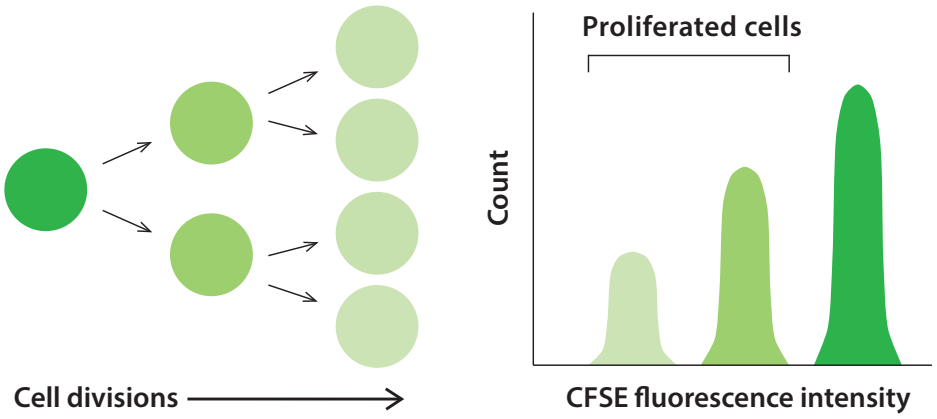

Fig. 14: CFSE is a fluorescent dye which binds to intracellular proteins, and is shared equally by daughter cells in every round of cell division. Thus, CFSE fluorescence is halved in each subsequent generation.

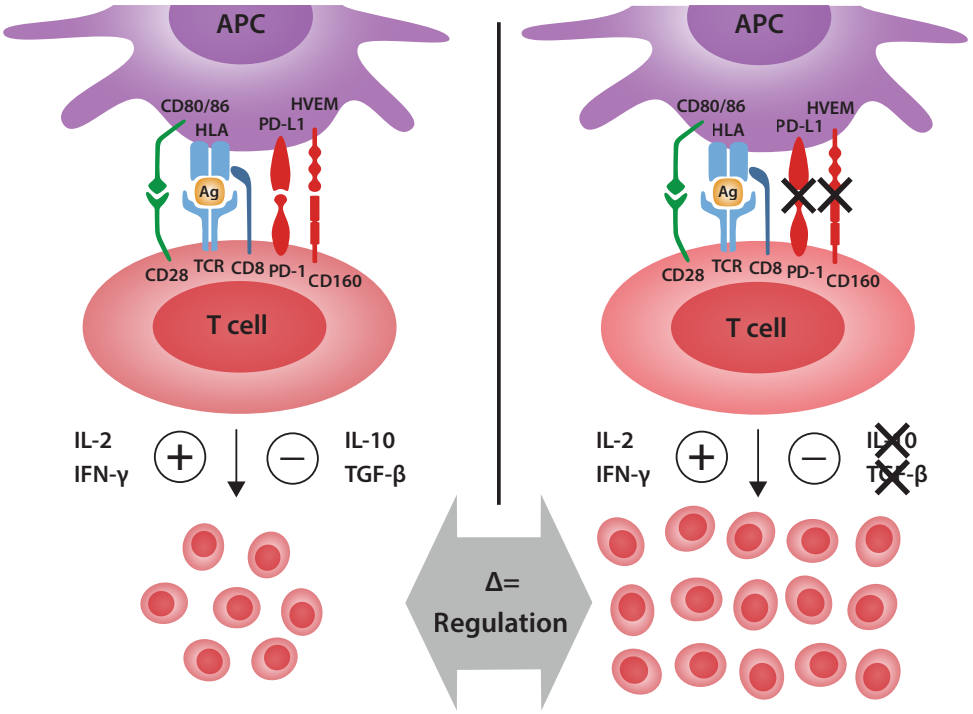

Fig. 15: Schematic of the regulation assay in paper II. T cell activation and proliferation is under tight control by co-stimulatory and co-inhibitory receptors, as well as pro- and anti-inflammatory cytokines (left panel). To quantify the negative regulatory component, we used monoclonal antibodies to block the inhibitory pathways (right panel). The difference in proliferation between the two culture conditions defines functional T cell regulation (FTR) in paper II. Modified from [281].

The monoclonal antibodies used to block inhibitory pathways and thus demonstrate regulation of $CD8^+$ T cell responses in paper II have all been used for similar applications in published studies [168, 329, 330].

3.4.3 Polyfunctional CD8 responses

While the proportion of $CD8^+$ T cells proliferating in response to an antigenic stimulus is an established in vitro functional parameter, the effector molecules expressed by individual $CD8^+$ T cells on stimulation also represent an important aspect of the adaptive immune response. As polychromatic flow cytometry has advanced, investigators have been able to quantify multiple effector functions in diverse lymphocyte subsets simultaneously. Betts et al. have shown that the HIV-specific $CD8^+$ T cells of patients who control their HIV infection in the absence of antiretroviral therapy express many effector functions concurrently [137], suggesting that not only the quantity but also the quality of the antiviral T cell response is of clinical significance.

In paper III, the effect of a COX-2 inhibitor on both the magnitude and quality of HIV Gag-specific $CD8^+$ T cell responses was evaluated in a short stimulation assay followed by intracellular staining of cytokines IFN-γ, TNF-α, IL-2 and MIP-1β and the membrane glycoprotein CD107a, which is a marker of degranulation [331].

3.4.4 Peptide stimulation

HIV-specific $CD8^+$ T cells were stimulated with Gag and Env peptide pools in paper II, and a Gag peptide pool in paper III. The peptides were 15-mers, overlapping by 11 amino acids. This allows direct binding by HLA class I molecules, and thus efficient stimulation of $CD8^+$ T cells [332]. The alternative, using whole viral proteins, would require antigen processing and presentation by APCs in the cultures. As these antigens, processed by the exogenous pathway, are presented primarily on HLA type II molecules, they stimulate $CD4^+$ T cells to a greater extent than $CD8^+$ T cells [333]. Gag peptides were chosen as stimulating antigens due to the many observations that Gag-specific $CD8^+$ T cell responses are associated with viral control in chronic HIV infection [128, 131, 334]. $CD8^+$ T cell responses toward Env do not seem to have the same clinical significance, but Env epitopes are the major target for broadly neutralising antibodies against HIV [290]. As $CD4^+$ T cell help is involved in B cell production of antibodies, regulation of CD4 responses to Env would have been relevant to examine. However, due to constraints in the number of cellular markers we could examine concurrently, only CD8 regulation was investigated in the study in paper II.

3.5 Statistical considerations

All data in the studies included in this thesis were analysed using non-parametric statistical methods. The rationale for this was primarily the observation that many of the biological variables studied did not follow a normal (Gaussian) distribution, and that sample sizes were relatively small. Non-parametric methods do not assume normal data, but as the most common non-parametric tests consider only ranks and ignore the numerical values, a lot of information is lost and they are generally less powerful for detecting differences. With larger sample sizes, parametric methods can perform well with non-normal data [335], but the limited number of patients included in the studies rendered parametric methods less suitable for our purposes.

As the studies discussed here were largely exploratory in nature, corrections were not made for multiple comparisons, by Bonferroni or other methods. This no doubt increased the likelihood of type I statistical errors, i.e. falsely rejecting the null hypothesis when it is true, or expressed more intuitively, of detecting differences where there were none. However, to avoid type II errors, and potentially missing important, hypothesis-generating observations, no corrections were made, and the threshold of statistical significance set at 0.05 throughout. Furthermore, a few observations with $p>0.05$ were reported as trends, and correspondingly were interpreted with caution.

4. Discussion

4.1 Paper I: Pre-ART MIP-1β as a marker of INR?

The pre-ART CD4 count is the strongest predictor of immunological non-response to suppressive ART, underlining the need to identify cases of HIV infection early and initiate treatment before advanced immunodeficiency is established. Despite this, some patients will continue presenting late to care. As only a minority of patients with low pre-ART CD4 counts become INR, other markers to identify patients at risk of INR may facilitate early intervention and improved outcomes. Our initial hypothesis was that high pre-ART levels of immune activation and inflammation could increase the risk of INR, and thus that one or more inflammatory markers in plasma might be of use to predict INR development.

In paper I, we stratified patients according to their CD4 count before ART initiation and after a median 8.4 years of follow-up, defining three groups;

- long-term INR: pre-ART CD4 <200 cells/μL, final CD4 <350 cells/μL
- low-CD4 immune responders (IR), pre-ART CD4 <200 cells/μL, final CD4 >350 cells/μL
- high-CD4 IR, pre-ART CD4 >200 cells/μL, final CD4 >350 cells/μL

As expected, no patient with a CD4 count above 200 cells/μL before ART had a CD4 count below 350 cells/μL in their final sample. Thus, in order to identify a marker which could provide additional information on INR risk, we decided to restrict the analysis only to patients with a low pre-ART CD4 count, i.e. compare long-term INR to low-CD4 IR. These two groups were similar in all baseline parameters except one, the plasma level of the chemokine MIP-1β, which was elevated in long-term INR pre-ART and throughout the first three years of ART.

This finding was somewhat unexpected, as in the study cohort as a whole, plasma MIP-1β in fact increased slightly after ART initiation. This was in contrast to levels of plasma IP-10, a sensitive but unspecific marker of inflammation, which fell considerably

after ART, though not to the level of HIV-negative controls. Our observations of higher MIP-1β and lower IP-10 after ART initiation are in keeping with other studies [336-339], and seem to imply that plasma MIP-1β does not simply reflect gross systemic inflammation, but represents some other facet of HIV immunopathology which may hamper CD4 reconstitution on ART.

Plasma MIP-1β has previously been positively associated with the severity of chronic inflammatory conditions such psoriasis and psoriatic arthritis [340, 341], and was elevated in experimental endotoxaemia, along with pro-inflammatory cytokine TNF-α [342]. However, a study which measured MIP-1β in the plasma of patients acutely infected with dengue virus found an inverse relationship with disease severity, in contrast to established pro-inflammatory mediators like IFN-γ and IL-1β [343]. Along with our data, these somewhat contradictory findings underscore our lack of understanding of the significance of circulating MIP-1β in various inflammatory conditions.

While in vitro studies have shown that MIP-1β can protect $CD4^+$ T cells from HIV infection by blocking and downregulating CCR5 [142, 143], this beneficial effect is likely to be minimal in ART-treated infection with suppressed viral replication. Conversely, the persistently elevated MIP-1β levels we observe in long-term INR patients in our study may instead contribute to continued hyperactivation of the $CD4^+$ T cell compartment. An inverse correlation between CCR5 expression on $CD4^+$ T cells and immune reconstitution on ART has been reported [344], supporting the notion that a CCR5 ligand like MIP-1β can promote INR development. Many immune cells have the potential to produce MIP-1β, including monocytes, DCs and NK cells [345-349], and the source of persistently elevated MIP-1β in plasma could be a particular subset remaining pathologically activated after the suppression of viraemia by ART.

As briefly discussed in paper I, implicating MIP-1β in INR development suggests a potential for CCR5 antagonists like maraviroc to improve immune reconstitution. Due to its targeting of a host chemokine receptor, immunomodulatory effects of maraviroc independent of its antiretroviral action have been postulated, and a reduction of T cell activation has been demonstrated in several pilot studies of maraviroc intensification of ART [240, 350, 351]. However, in the only placebo-controlled study performed to date, maraviroc intensification actually increased activation of T cells both in rectal lymphoid tissue and peripheral blood, as well as monocyte activation markers sCD14 and sCD163 [352]. As the CCR5-MIP-1β complex is internalised after binding, CCR5 blockade not surprisingly led to an increase in serum MIP-1β in the same study. Thus, the authors

propose that MIP-1β and other β-chemokines like MIP-1α and RANTES (regulated on activation, normal T-cell expressed and secreted) may be driving immune activation via their other cognate receptors, which are not blocked by maraviroc. Should this be the case, maraviroc may in fact have a detrimental immunological impact in INR with elevated plasma MIP-1β.

So far, none of the trials of maraviroc intensification of ART have shown improved CD4 reconstitution when viraemia is already suppressed to below 50 copies/mL. However. in these studies, patients have not been stratified based on pre-treatment MIP-1β. Whether baseline plasma levels of MIP-1β can predict the immunological effects of maraviroc intensification of ART should potentially be addressed in a future trial.

There are methodological issues in paper I which should be discussed, and may influence the interpretation of our data. The study was retrospective, introducing the risk of bias when selecting the cohort to be examined and defining the responder groups to be compared. Ideally, the cohort would have been included in a prospective manner, with the ART response groups defined in advance, as part of the study protocol. However, as scientific questions and the methods to investigate them arise, analysis of existing biobanked material enables a far more rapid test of hypotheses. Furthermore, the responder groups in our study were defined before the cytokine data was examined, using CD4 count thresholds commonly used at the time to define severe immunocompromise (<200 cells/μL) and adequate response to treatment (>350 cells/μL). Nonetheless, the relationship between plasma MIP-1β and INR development should be confirmed in a larger, prospectively sampled cohort.

As discussed in the paper and in section 3.2.1, we recognised that cryopreservation of plasma samples at -20°C for up to a decade was suboptimal. We addressed this issue by running samples from both an HIV-positive comparator cohort and HIV-negative blood donors, stored at -70°C, in the same analysis runs. This allowed the identification of significantly degraded markers in our study samples, and these analytes were excluded from further analysis. This information is potentially useful for other groups analysing plasma samples stored under suboptimal conditions. Importantly, no significant degradation of MIP-1β was apparent after storage at -20°C.

As discussed in section 3.5, no corrections for multiple comparisons were made in the statistical analysis of the data, risking type I statistical errors. However, the consistency of our data, with MIP-1β significantly elevated at several time points and in regression analysis, supports the validity of our observations.

4.2 Paper II: Assay to assess regulation of HIV-specific CD8$^+$ T cell responses.

Chronic HIV infection induces potent immunoregulatory mechanisms, which include an increased expression of regulatory cytokines and inhibitory receptors. These regulatory pathways contribute to functional impairment of HIV-specific CD8$^+$ T cells, which has in turn been linked both to untreated HIV disease progression and an inability to clear viral reservoirs in cure strategies.

In several studies, our group has explored a simple in vitro assay to quantify the functional suppression of T cells by regulatory cytokines IL-10 and TGF-β [281, 282, 353]. This was motivated initially by observations made in trials of the therapeutic vaccine Vacc-4x, which is based on four modified Gag peptides, and designed to induce HIV-specific CTL clones. While most patients responded to initial immunisation with durable vaccine-specific CD8 proliferative responses [272, 354], repeated booster doses progressively compromised cellular responses to vaccine peptides in a significant proportion of patients [281]. When these samples were restimulated in the presence of blocking mAbs to IL-10 and TGF-β, the response was significantly improved, suggesting an induction of regulatory mechanisms by booster immunisation. In a trial of nasal Vacc-4x administration, an association was found between pre-existing regulation and poor pre-immunisation responses to Vacc-4x peptides [282]. However, the same pre-existing regulation actually correlated positively with vaccine efficacy, suggesting that vaccine responses may be regulated differently depending on immunisation modality.

While the two aforementioned regulation studies explored vaccine antigen-related regulation in ART-treated patients, the same assay, using HIV Gag and Env consensus peptide pools, has also been previously tested in ART-naïve patients [353]. This study found considerable heterogeneity in regulation measured in this way, with large variability between patients. There was no clear relationship between HIV peptide-induced activation and the related IL-10/TGF-β-mediated regulation, nor between regulation in Gag- and Env-stimulated cultures. However, the HIV antigen-related regulation parameter seemed to have some relation to clinical progression of untreated infection, as patients with regulation to both Gag and Env antigens were characterised by lower CD4 counts and higher CD4 loss rates. Patients with little or no observed regulation had the most favourable clinical profile, while patients with Gag-associated regulation only were in an intermediate clinical state.

With these observations in mind, the study in paper II was initiated, in which the regulation assay was expanded to include inhibitory receptors PD-1 and CD160. Once again, substantial heterogeneity was found between patients and HIV antigens in the magnitude of regulation detected. However, there was some concordance between regulation mediated by different pathways, although due to the small number of patients with detectable regulation these relationships were not statistically significant. Such concordance is unsurprising, as we might expect T cells specific for given HIV antigens to be subject to regulation by several mechanisms concurrently in chronic infection. This is in accordance with the numerous studies showing a cumulative and/or synergistic dysfunction of T cells expressing multiple inhibitory molecules [166, 168, 355-357].

In order to further characterise the contribution of various inhibitory pathways to functional regulation of T cells, the blocking mAbs were added singly and in various combinations to the cultures. As discussed in the paper, we observed synergism between IL-10 and PD-1 blockade only on Gag stimulation, while the presence of TGF-β seemed necessary for IL-10 to exert any suppressive effect on Env-stimulated cells. Regulation mediated by interaction of CD160 with its ligand HVEM was detected only in two patients, and in these subjects no other regulation was observed. While we cannot draw conclusions about the functional independence of the CD160 pathway from observations in so few patients, CD160-mediated suppression of $CD8^+$ T cells independently of PD-1 expression has been observed in other viral infections [358].

As we had previously observed an association between regulation to given antigens and clinical variables [353], patients were once again grouped based on their regulatory phenotype. For this purpose we used the blocking conditions which identified the greatest number of regulator patients, i.e. IL-10/PD-1 for Gag regulation and IL-10/TGF-β for Env regulation. The least favourable clinical profile was then exhibited by patients with isolated Env regulation, which is in accordance with unpublished data from previous trials (D. Kvale, personal communication). However, while Env-specific CD8 responses have been associated with higher viral loads in untreated infection [129], the three initial Env regulators in our study had proliferative Env responses of low magnitude in unblocked cultures. Pan regulators, in the other hand, with the highest proliferative responses to both Gag and Env, also had the most favourable clinical parameters. Non-regulators were clinically heterogeneous, but were clearly characterised by the poorest proliferative responses to both Gag and Env.

These observations do not conform to the most intuitive conceptualisation of regulation, according to which we would expect an inverse correlation between antigen-induced proliferation and our regulation parameter. However, one could speculate that a powerful antigen-driven proliferative drive in fact induces higher expression of inhibitory receptors and regulatory cytokines in culture, thereby also increasing the potential for mAb blockade of those inhibitory pathways to further increase proliferation. This highlights an as yet unanswered question in our exploration of T cell regulation in HIV: to what extent the regulatory mechanisms we investigate are present before we stimulate the cells in vitro.

This issue has consequences for perhaps the most relevant clinical application of an assay such as this: to predict the clinical efficacy of blocking these inhibitory pathways. This approach, often referred to as immune checkpoint blockade, is currently revolutionising oncology, and a number of in vitro and non-human primate studies suggest that a similar strategy may hold great potential for improving HIV-specific immunity, particularly in an HIV cure setting [168, 279, 359-361]. However, in both clinical cancer trials and preclinical studies in chronic viral infections, response rates to immune checkpoint blockade are highly variable, and there is a paucity of biomarkers that can predict which patients or study animals will benefit from this type of immunotherapy [170, 171]. We have hypothesised that an assay such as ours might potentially be used to identify patients in which immune checkpoint blockade will have the highest likelihood of success.

To this end, several additional analyses would have strengthened the study in paper II. The expression of PD-1 and its ligands on both effector T cells and target cells is an obvious putative biomarker predictive of response to anti-PD-1 therapy [170-172]. Determining the relationship between pre-existing PD-1 expression on T cell subsets, antigen-induced proliferation and PD-1-mediated regulation might have provided an indication of whether our regulation data is a more useful predictor of anti-PD-1 therapy response. Similarly, although the clinical feasibility of IL-10 blockade is uncertain and TGF-β inhibition is further from clinical use than PD-1 blockade [172], relating cytokine concentrations both in plasma and in stimulated culture supernatants to antigen-stimulated proliferation and related regulation would have provided novel information about the utility of our assay.

Several methodological issues in this study merit discussion. Firstly, as our assay is clearly dependent on viable T cells capable of stimulation and proliferation, we checked all cells for viability after thawing, used a viability dye in the flow cytometric analysis and had

a positive control culture in all experiments. However, the proportion of non-regulator patients with poor proliferative responses in both unblocked and mAb-blocked cultures raises the question of whether this was not due to functional compromise in vivo, but to problems related to sample processing and storage.

Secondly, the study would clearly also have benefited from a more systematic validation of the regulation assay. While preliminary experiments included repeated analysis of certain samples, giving consistent results, the assay should ideally have been performed in duplicate or triplicate, to assess the intra-assay coefficient of variability, i.e. the precision of our data. In this study, such a validation process was limited by available patient PBMC, but follow-up studies of regulation assays must include such assessments.

Finally, the choice to continue our exploration of T cell regulation in ART-naïve as opposed to ART-treated patients should be commented upon. As discussed in section 1.6.4.4, HIV-specific T cell responses, at least as measured by in vitro assays, are diminished after the initiation of ART. Our results in preliminary experiments, analysing samples from both ART-naïve and ART-treated patients, were in keeping with this, with substantially more vigorous HIV peptide-induced responses in ART-naïve patients. Unsurprisingly, the corresponding regulation effects in ART-naïve patient samples were also of a larger magnitude. As we assumed a certain assay-related variability, ART-naïve patients were selected for our initial characterisation of regulation effects in order to achieve as high a "signal-to-noise" ratio as possible. That said, as touched upon in the paper, ART-treated HIV infection is rapidly becoming the norm, and candidates for future HIV cure interventions will probably all be virally suppressed on ART. Thus, future regulation assay studies should absolutely include ART-treated patients, provided the assay is suitably validated, as discussed above.

4.3 Paper III: Immunological effects of COX-2i in HIV infection

HIV infection is characterised by a chronic hyperactivation of both innate and adaptive immune cells, and this immune activation and associated inflammation has been implicated both in the development of immunodeficiency and excess morbidity from non-AIDS conditions. Considering the multitude of negative feedback loops in place to rein in the immune response, it is unsurprising that pathologically increased activation of these cellular subsets may induce functional deficits. As discussed in section 1.5.11, we have hypothesised that enhanced activity of the COX-2–PGE_2 axis, possibly driven

by microbial translocation from the gut, contributes to T cell inhibition in HIV infection, and that a COX-2 inhibitor may both attenuate immune activation and improve T cell function in HIV-infected patients.

While we have previously demonstrated reduced CD38 expression on T cells in ART-naïve patients receiving high-dose celecoxib [209], both ART-naïve and ART-treated patients with a suboptimal CD4 count were included in this follow-up study, as detailed in paper III. This was motivated by several observations. Firstly, as treatment is now recommended for all HIV-infected patients and ART coverage improves worldwide, the clinical relevance of ART-treated HIV infection surpasses that of untreated infection in most settings. Furthermore, while HIV-associated immune dysregulation is ameliorated when viral replication is suppressed by ART, many markers of immune activation and inflammation do not reach the level of HIV-uninfected controls even after years of stable ART [227, 337, 339], particularly in immunological non-responders [122]. Finally, the reduction in immune activation after ART initiation is not matched by a corresponding increase in HIV-specific CD8 responses [153, 269, 270]. This suggests a therapeutic potential of anti-inflammatory drugs, including COX-2i, also in ART-treated patients. However, as touched upon in section 1.6.3, reducing residual immune activation in ART-treated infection has proved challenging in many trials.

In this study, potentially beneficial effects on both the activation status and HIV Gag-specific effector responses of $CD8^+$ T cells, as well as on thrombin generation, were observed in ART-naïve patients. However, corresponding effects on plasma markers of inflammation were absent in this patient group. As innate immune cells such as monocytes, macrophages and dendritic cells are the main source of these inflammatory mediators, of which several activate both T cells [362] and the coagulation system [363], this implies that the COX-2i effect of reduced eicosanoid synthesis primarily elicits its action downstream of innate immune activation. This hypothesis is further supported by the fact that no convincing effect of etoricoxib was observed on IDO activity, considering that IDO is induced primarily in innate immune cells [192].

As discussed in the paper, the effects we did observe on $CD8^+$ T cell activation and Gag-induced CD8 effector responses in vitro were modest, which may be related to COX-2i dosage. The high dose of celecoxib given in our previous study was associated with a relatively high incidence of rash, and so the use of COX-2i at doses with an appreciable immunomodulatory effect in HIV infected patients may not be feasible due to side-effects.

In ART-treated patients with suboptimal CD4 counts, by contrast, we did not observe any significant effects of etoricoxib at all. Again, dosage may have played a role, as the 90 mg qd of etoricoxib administered in this study may not have been sufficient to further reduce residual immune activation and inflammation in patients on stable ART. Moreover, our study may have been underpowered to detect modest COX-2i effects. Challenges in both patient inclusion and follow-up contributed to an unfortunately low number of patients in each treatment arm, which was stipulated to be at least 15 according to the initial study protocol. Had the study recruited this number of patients, the random differences we observed in baseline CD4 counts and CD4/CD8 ratio may have been less acute, and not prompted the exclusion of patients to ensure a valid analysis of COX-2i effects. We could also reasonably have anticipated that baseline differences in CD4 count large enough to complicate endpoint analysis might occur randomly in this small trial. This problem could have been ameliorated by incorporating stratification on baseline CD4 count into the randomisation procedure [364]. Alternatively, the baseline CD4 count could have been incorporated into a multivariate analysis to elucidate its effect on the immunological outcomes, but this was again hampered by a small number of patients.

A second major goal of the study was to investigate the effect of COX-2i on antibody responses to T cell-dependent vaccines. B cells are also subject to HIV-induced polyclonal activation, and hyperglobulinaemia is common in HIV-infected patients, at least in the absence of ART [365, 366]. However, as is the case with T cells, this hyperactivation of B cells is associated with functional defects, and impaired responses to both T cell-dependent influenza and tetanus toxoid and T cell-independent pneumococcal polysaccharide vaccines have been observed [367-369]. We have hypothesised that COX-2i therapy may not only improve T cell responses on the CTL/Th1 axis, but potentially also Th2 help of B cells, and thereby improve antibody responses to T cell-dependent vaccines. Our previous celecoxib study provided some support for this notion, with significantly improved IgG responses to tetanus toxoid vaccination, but only when the analysis was limited to patients with evidence of previous vaccination at baseline.

Unfortunately, we detected no such effect of COX-2i therapy on vaccine IgG responses in this trial. In fact, there were some indications of the opposite effect, as patients receiving etoricoxib for 5 weeks before and 4 weeks after vaccination seemed to have the poorest antibody responses to both tetanus and conjugated pneumococcal vaccines. Potential explanations for this are discussed in the paper, although once again, small group sizes hampered statistical analysis and our ability to draw firm conclusions from this trial.

Were this trial to be repeated, a more robust sample size as determined by a sound power calculation would have been the most important improvement. While our primary endpoints would be similar, several additional analyses might shed further light on the effects of COX-2i in these patients. As we assume that innate immune cells excrete the majority of COX-2-derived eicosanoids, a characterisation of circulating monocytes and dendritic cells would be justified, particularly with regard to activation status, COX-2 expression and perhaps in vitro eicosanoid secretion in response to TLR agonists. Circulating PGE_2 levels should also be quantified, considering our hypothesis that this eicosanoid plays a central role in COX-2-related immunomodulation.

COX-2i effects on humoral immunity should, given our discrepant results thus far, be investigated further. In future trial, examining the phenotype and functional characteristics of circulating B cells may aid our understanding of potential COX-2i effects on antibody responses to vaccines. Considering the increasing momentum in the HIV vaccine field, also in relation to cure research, improved vaccine responses could be the most clinically significant effect of COX-2i in HIV-infected patients.

Finally, an important caveat of all these investigations is that they solely examine the cells and signalling molecules present in the circulation. The determinant events of HIV infection largely take place in secondary lymphoid tissue, in GALT and in lymph nodes, which unfortunately are much less available for sampling than the blood. In a well-designed, adequately powered trial, some form of lymphoid tissue sampling would be desirable, in order to examine both T cells, B cells and innate immune cells in the most physiologically relevant setting.

5. Conclusions

This work was undertaken to investigate certain hypotheses concerning how inflammation impacts on HIV pathogenesis and how these effects can be modulated for the benefit of HIV-infected patients. From the three hypotheses raised in this thesis, certain tentative conclusions can be drawn:

1. *Immunological non-response to ART can be predicted by plasma markers of inflammation measured before ART initiation.*

Pre-ART plasma levels of the chemokine MIP-1β may be a useful biomarker predictive of immunological non-response to ART. Elevated plasma levels of MIP-1β observed before and during ART in long-term immunological non-responders may represent a facet of HIV-associated immunopathology contributing to the development of immunological non-response to ART. This effect does not seem directly dependent on generalised immune activation and inflammation.

2. *Functional regulation of HIV-specific T cell responses can be quantified in an in vitro assay.*

HIV-specific T cell function, as determined by peptide-stimulated proliferation, is regulated by cytokines IL-10 and TGF-β and co-inhibitory molecule PD-1, with a high degree of inter-individual variability. This type of functional regulation assay may be particularly relevant for predicting response to immune checkpoint blockade. However, the assay must be validated further before this can be confirmed in clinical trials of immunotherapy.

3. *A cyclooxygenase (COX) 2 inhibitor may reverse prostaglandin E_2-dependent inhibition of T cell functions and reduce generalised hyperactivation of T cells in HIV-infected patients both off and on ART.*

A COX-2i has the potential to beneficially modulate immune activation, T cell immunity and the coagulation system in untreated HIV infection. Whether these effects are clinically significant is still uncertain. No evidence was found for a role of COX-2i in ART-treated infection.

6. Future perspectives

While the global rollout of ART steadily reduces AIDS-related mortality, the incidence of new HIV infections continues to outpace the number of patients commencing ART. We can thus expect the global population of people living with HIV to grow for many years to come. Hopefully, continued efforts to attenuate HIV-associated inflammation and improve immune reconstitution in ART-treated infection will contribute to improving the health of people living with HIV. However, this population may still be at increased risk of non-AIDS morbidity, and the combined cost of providing ART and care for these HIV-related co-morbidities will severely strain the health systems of many high-burden countries.

A functional cure strategy able to induce durable viral control in the absence of ART, and which is also scalable to high-burden, low-resource settings, would massively contribute to reducing the medical, social and economic burden of HIV. While this branch of research is gaining significant momentum, no potential therapeutic approach has so far progressed beyond the proof of concept stage, and it will take many years before any significant clinical impact can be made by cure interventions. In the meantime, early diagnosis and ART initiation has the potential to reduce immune activation and the size of the latent viral reservoir [287-289], perhaps facilitating future cure interventions, while at the same time preventing further HIV transmission [78, 79].

However, although antiretroviral treatment as prevention (TasP), pre-exposure prophylaxis (PrEP) and prevention of mother-to-child transmission (PMTCT) are vital preventive measures, a broader approach must be taken to address risk factors for HIV acquisition. In many countries severely affected by the HIV pandemic, adolescent girls and young women are at significantly increased risk for HIV acquisition, in part due to gender inequality and rife sexual violence [370-372]. Improving attitudes to women and increasing their control in sexual relations has a considerable preventive potential in these settings [370].

Finally, the Holy Grail of HIV prevention is the development of an effective prophylactic vaccine. While an error-prone reverse transcriptase, frequent in vivo recombination

and extensive glycosylation and variability of the Env spike have hampered HIV vaccine efforts thus far, recent insights into the three-dimensional structure of Env and of multiple broadly neutralising antibodies have reinvigorated the field [290, 373]. Immunisation strategies to elicit such antibodies in vivo are being intensively investigated.

In conclusion, after three and a half decades, the knowledge and tools at our disposal provide hope for an end to the HIV/AIDS pandemic. Achieving this goal, however, will require an extensive provision of antiretroviral drugs for treatment and prevention, as well as the implementation of other evidence-based preventive measures, and will be dependent on considerable political will. For its part, the scientific community must continue to strive for improved care of people living with HIV. This will undoubtedly include addressing the residual immunological dysfunction in ART-treated infection, but may hopefully also, given a concerted and persistent effort, yield the possibility of ART-free HIV remission at some point in the future.

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RESEARCH ARTICLE

Regulation of Gag- and Env-Specific CD8$^+$ T Cell Responses in ART-Naïve HIV-Infected Patients: Potential Implications for Individualized Immunotherapy

Christian Prebensen[1,2,3], Andreas Lind[1], Anne-Ma Dyrhol-Riise[1,2,3], Dag Kvale[1,2,3]*

1 Department of Infectious Diseases, Oslo University Hospital, Oslo, Norway, **2** Institute of Clinical Medicine, University of Oslo, Oslo, Norway, **3** K.G. Jebsen Inflammation Research Centre, University of Oslo, Oslo, Norway

* dag.kvale@medisin.uio.no

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Abstract

Strategies to develop a functional cure for HIV infection will likely require boosting of effector T cell responses to eliminate reactivated, latently infected cells. We have recently explored an assay for assessing antigen-specific regulation of T cell proliferation, which was related to clinical progression in untreated patients and to vaccine efficacy in two trials of therapeutic Gag-based vaccines. We here expand the same assay to further investigate regulation mediated by various inhibitory pathways. Peripheral blood mononuclear cells from 26 asymptomatic HIV-infected, antiretroviral therapy-naïve patients were stimulated with Gag and Env overlapping peptide panels for 5 days. Monoclonal antibodies (mAbs) blocking inhibitory mediators interleukin (IL) 10, transforming growth factor (TGF) β, programmed death ligand (PD–L) 1 and herpes virus entry mediator (HVEM) were added to parallel cultures. Functional T cell regulation (FTR) was defined as the difference in proliferation between stimulated cultures with and without blocking mAbs. FTR was detected in 54% of patients. Blockade of IL-10/PD-L1 and IL10/TGF-β detected all cases with Gag- and Env-associated FTR, respectively. In accordance with previous findings, isolated Env FTR was associated with higher plasma HIV RNA and lower CD4 counts, while patients with both Gag and Env FTR also had higher Gag- and Env-specific proliferative CD8$^+$ T cell responses. There was no association between FTR and frequencies of activated regulatory T cells. In conclusion, we observed substantial heterogeneity in FTR between patients, inhibitory pathways and HIV antigens. FTR may help to individualize immunomodulation and warrants further assessment in clinical immunotherapy trials.

Introduction

Effective HIV-specific cytotoxic T lymphocyte (CTL) responses are central to immune control of HIV infection [1,2]. CD8$^+$ T cell responses against HIV emerge during the course of acute

infection, concurrently with falling plasma viremia [3,4]. The small minority of patients who naturally control HIV infection maintain highly effective HIV-specific CTL responses over time, exhibiting both polyfunctionality and potent HIV-suppressive effects [5,6]. On the other hand, most patients chronically infected with HIV progressively lose HIV-specific $CD8^+$ T cell responses [7], through reduced $CD4^+$ T cell help [8], clonal T cell loss [9], immune exhaustion [10] and other negative regulatory mechanisms. Importantly, these defects in HIV-specific T cell immunity are not fully restored by antiretroviral therapy (ART) [11,12].

Despite its ability to durably suppress HIV replication, ART does not eradicate the latent viral reservoir, and lifelong therapy is necessary to avoid rapid viral rebound [13]. This has sparked efforts to develop therapeutic strategies able to establish durable viral control in the absence of ART, a so-called functional cure [14,15]. Many of these approaches will require an induction or boosting of the patients' impaired HIV-specific CTL function in order to eliminate reactivated, latently infected cells [16] and maintain viral control. This may be attained by therapeutic vaccination or other immunomodulatory therapy.

Antigen-induced T cell activation and proliferation are subject to negative regulation through a variety of signalling pathways, including the anti-inflammatory cytokines interleukin (IL) 10 and transforming growth factor (TGF) β as well as negative co-signalling molecules programmed death (PD) 1 and CD160. We have recently explored an assay for assessing negative regulation of HIV-specific T cell function mediated by IL-10 and TGF-β. This regulation parameter was associated with clinical progression in untreated HIV infection [17]. Moreover, pre-existing and evolving regulation of HIV vaccine-specific $CD8^+$ T cell responses coincided with low final responses against therapeutic Gag peptide-vaccines in ART-treated patients [18,19]. Thus, such an approach of assessing antigen-induced T cell regulation may prove clinically useful and these data suggest that regulation should be taken into account when considering patients for immunomodulatory therapy as part of a functional cure. In addition, quantifying the contribution of various pathways in suppressing T cell function may allow individually tailored interventions directed at these mechanisms [20–23].

The aim of this study was to further explore mechanisms of functional T cell regulation (FTR) of $CD8^+$ T cell responses against HIV Gag and Env antigens, mediated by not only IL-10 and TGF-β, but also PD-1/PD-L1 and CD160/HVEM pathways. We observed substantial heterogeneity in FTR between patients, inhibitory pathways and HIV antigens, and an apparent detrimental effect on clinical parameters of isolated Env-related FTR.

Methods

Study participants

Twenty-six asymptomatic HIV-1 seropositive patients from the Department of Infectious Diseases, University Oslo Hospital were included in the study. All included patients were above 18 years of age, ART-naïve and none fulfilled the criteria of elite or viremic controllers. No patients were co-infected with hepatitis B or C virus and none had symptoms or findings of intercurrent or opportunistic infections. $CD4^+$ and $CD8^+$ T cell counts and plasma HIV RNA were determined by routine clinical assays. Clinical characteristics are shown in Table 1.

The study was approved by The Norwegian South-Eastern Regional Committee for Medical and Health Research Ethics. Written, informed consent was obtained from all study participants.

Samples

Peripheral blood mononuclear cells (PBMC) were isolated using Vacutainer Cell Preparation Tubes (Becton Dickinson (BD), NJ, USA). Cells were frozen in RPMI containing 40% FCS and

Table 1. Clinical characteristics (n = 26).

Age (years)	42.5 (40.0–49.0) *
Gender (male/female)	23/3
Time since HIV diagnosis (years)	2.0 (1.0–3.9)
$CD4^+$ T cell counts (cells/uL)	416 (337–514)
$CD8^+$ T cell counts (cells/uL)	1 325 (1 054–1 611)
Plasma HIV RNA (copies/mL)	34 500 (10 000–81 000)

*: Data given as median (lower—upper quartile)

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10% DMSO and stored at -145°C. $CD4^+$ and $CD8^+$ T cell counts and plasma HIV RNA loads were determined by routine clinical assays.

Proliferation/Regulation assays

Cells were thawed, washed and reconstituted in AIM V serum-free medium (Life Technologies, Oslo, Norway) containing 0.1% human serum albumin. After an overnight rest, cells were pulse-labelled with *carboxyfluorescein diacetate succinimidyl ester* (CFSE, Life Technologies) at a concentration of 2 μM for 5 minutes. The following blocking antibodies were added to parallel culture wells at a final concentration of 10 μg/mL: anti-IL-10 (R&D Systems, MN, USA; clone 23738), anti-TGF-β (R&D; clone 1D11), anti-PD-L1 (eBioscience, CA, USA; clone MIH1), and anti-HVEM (R&D; clone 94801).

After a 30 minute incubation, cultures were stimulated with either Gag or Env 15-mer overlapping peptide panels (NIH AIDS Research and Reference Reagent Program, MD, USA) at a final concentration of 2 μg/mL/peptide. Staphylococcal Enterotoxin B (SEB, Sigma-Aldrich, MO, USA) at a final concentration of 0.5 μg/mL was used as a positive control.

Cells were cultured at 37°C in 5% CO_2 for 5 days, harvested, and stained with the following fluorochrome-conjugated antibodies: CD3 V450, CD8 APC-H7, HLA-DR BV605, CD45RA APC (all BD) and CD25 PE (Biolegend). 7-aminoactinomycin D (7-AAD, BD) was added for dead cell exclusion. Flow cytometry data were acquired on a BD FACS Canto II with BD Diva 6.1 software, and analyzed in FlowJo X (FlowJo LLC, OR, USA).

Gating, readouts and parameter definitions

Antigen-specific, proliferated $CD8^+$ T cells were defined by CFSE fluorescence between the second and sixth generation of $CFSE^{dim}$ cells (Fig 1A). The first generation was omitted based on the assumption that these late proliferating cells more likely represent unspecific bystander proliferation. Activated $CD8^+$ T cells were defined by co-expression of CD25 and HLA-DR. Activated regulatory T cells (aTregs) were defined by the phenotype $CD3^{+-}$ $CD8^-CD25^{hi}CD45RA^-$ [24]. In the absence of discrete cell populations, gating cut-offs were determined by the Fluorescence Minus One (FMO) method. Examples of all gates are given in S1 Fig.

Proliferative responses were defined as the difference in $CFSE^{dim}$ $CD8^+$ T cell fractions between antigen-stimulated and unstimulated cultures. Functional T cell regulation (FTR) was defined as the difference in the percentage of $CFSE^{dim}$ $CD8^+$ T cells between stimulated cultures with and without blocking antibodies, respectively (Fig 1A). In addition, a lower threshold of 0.5% was set to signify FTR beyond potential intra-assay variability, as we observed occasional negative FTR values between 0 and -0.5% (excluding a few outliers). FTR values $>$0.5% were consistently found on repeated analysis of selected samples.

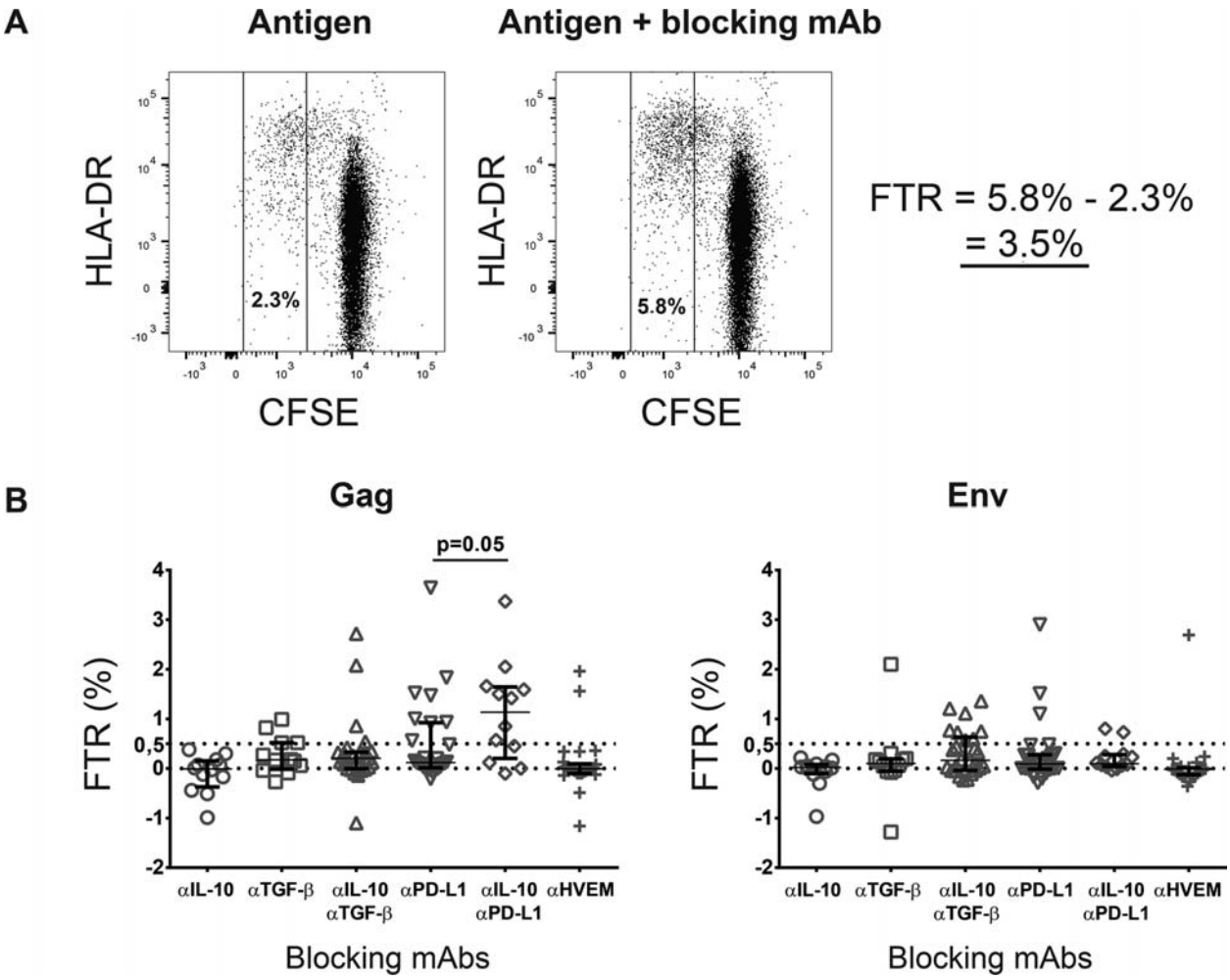

Fig 1. Functional T cell regulation (FTR) of CD8⁺ T cells from HIV-infected patients. (A) FTR as the difference between proliferating ($CFSE^{dim}$) $CD8^+$ T cells, in HIV antigen-stimulated cultures with and without blocking antibodies. Proliferating cells also express the activation marker HLA-DR. (B) Gag and Env FTR, assessed by single or dual blockade of inhibitory pathways. Median and upper/lower quartiles indicated. FTR defined as an increase in proliferating cells above a threshold of 0.5% (indicated by upper dashed line). P-value derived from paired test.

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Statistics

All statistical methods used were non-parametric. Comparisons between groups were assessed by Kruskal-Wallis and Mann-Whitney U tests. Correlations between parameters were assessed by Spearman rank sum tests. Paired data was assessed by Wilcoxon matched pair tests. Analyses were performed in SPSS v. 21 (IBM Corp. Armonk, NY) and Graphpad Prism 6 (Graphpad Software, La Jolla, CA). P-values below 0.05 were considered statistically significant.

Results

Heterogeneity in Gag and Env regulation

Samples from all patients were first assessed for $CD8^+$ FTR mediated by IL-10/TGF-β, the PD-1/PD-L1 and the CD160/HVEM pathways, respectively. Overall, 14 patients (54%) had

detectable FTR. In a subgroup of 12 patients who had quantifiable Gag and/or Env FTR detected by IL10/TGF-β dual blockade, we repeated the experiments with single blockade of IL-10 and TGF-β, to quantify the individual regulatory contribution of these two cytokines. In addition, we investigated the FTR effects of PD-L1, IL-10/PD-L1 and HVEM blockade in Gag and Env-stimulated cultures, respectively (Fig 1B).

We observed considerable heterogeneity, not only between Gag and Env FTR, but also between the regulatory effects of the different inhibitory pathways (Fig 1B). For example, we found no Gag or Env FTR mediated by IL-10 alone, while four patients had Gag FTR mediated by TGF-β alone, one of which also had Env FTR mediated by TGF-β. However, Env FTR assessed by IL-10/TGF-β dual blockade was observed in eight patients, suggesting additive or synergistic effects of the two cytokines on Env responses. For Gag FTR, an additive effect was obtained by blocking IL-10 in combination with PD-L1 ($p = 0.05$), but no such synergy was found for Env FTR (Fig 1B).

Blockade of the CD160 ligand HVEM identified only two patients with Gag FTR and another patient with Env FTR. No clear associations were found between HVEM FTR assessments and FTR obtained by the other inhibitory pathways.

Taken together, substantial inter-individual differences were noted for FTR in relation to both signalling pathways and HIV antigens.

Different blocking conditions best identify patients with Gag and Env FTR

Because we hypothesized that HIV antigen-specific FTR is important to quantify in a clinical immunotherapy setting, we next selected the assay conditions which revealed the highest number of patients with quantifiable Gag and Env FTR, respectively. Notably, dual blockade of IL-10/PD-L1 revealed all patients with Gag FTR, whereas combined blockade of IL-10/TGF-β identified all patients with Env FTR (Fig 2).

The heterogeneity among patients who had detectable FTR was once again exemplified by there being no significant concordance between Gag and Env FTR mediated by the same signalling pathways (data not shown).

Regulation associated with viremia and CD4 counts

Despite the heterogeneity in FTR by signal pathways and antigens, patients were tentatively grouped on the basis of whether they exhibited FTR associated with Gag only (Gag-regulators, n = 6), Env only (Env-regulators, n = 3), both Gag and Env (Pan-regulators, n = 5), or neither Gag nor Env (Non-regulators, n = 12). There were no differences in age or time since HIV diagnosis between the regulator subgroups. While the paucity of Env-regulators hampered statistical analysis, this group nevertheless had lower CD4 counts ($p = 0.03$) and higher viral loads ($p = 0.05$) than Pan-regulators (Fig 3A and 3B). All Env regulators had a low CD4/CD8 ratio, although there were no differences between groups (Fig 3C). Pan-regulators, on the other hand, had higher CD4 counts ($p = 0.02$) and higher both Gag- and Env-specific proliferative responses than Non-regulators ($p = 0.02$ and $p = 0.04$, respectively) (Fig 3D and 3E).

In order to confirm these observations, in particular the characteristics of more rapidly progressive HIV disease exhibited by Env-regulators, we reanalyzed $CD8^+$ T cell Gag and Env FTR in another cross-sectionally sampled HIV cohort of 23 ART-naïve viremic patients. This supplementary cohort had similar clinical characteristics to the study cohort ($CD4^+$ T cell count 425 (255–549) cells/uL; $CD8^+$ T cell count 1222 (771–2019) cells/uL; plasma HIV RNA 52 000 (13 000–130 000) copies/mL; n.s.). When regulator subgroups were compared in the two cohorts combined (n = 49; 13 Gag-, 5 Env-, 6 Pan-, and 25 Non-regulators), we found that

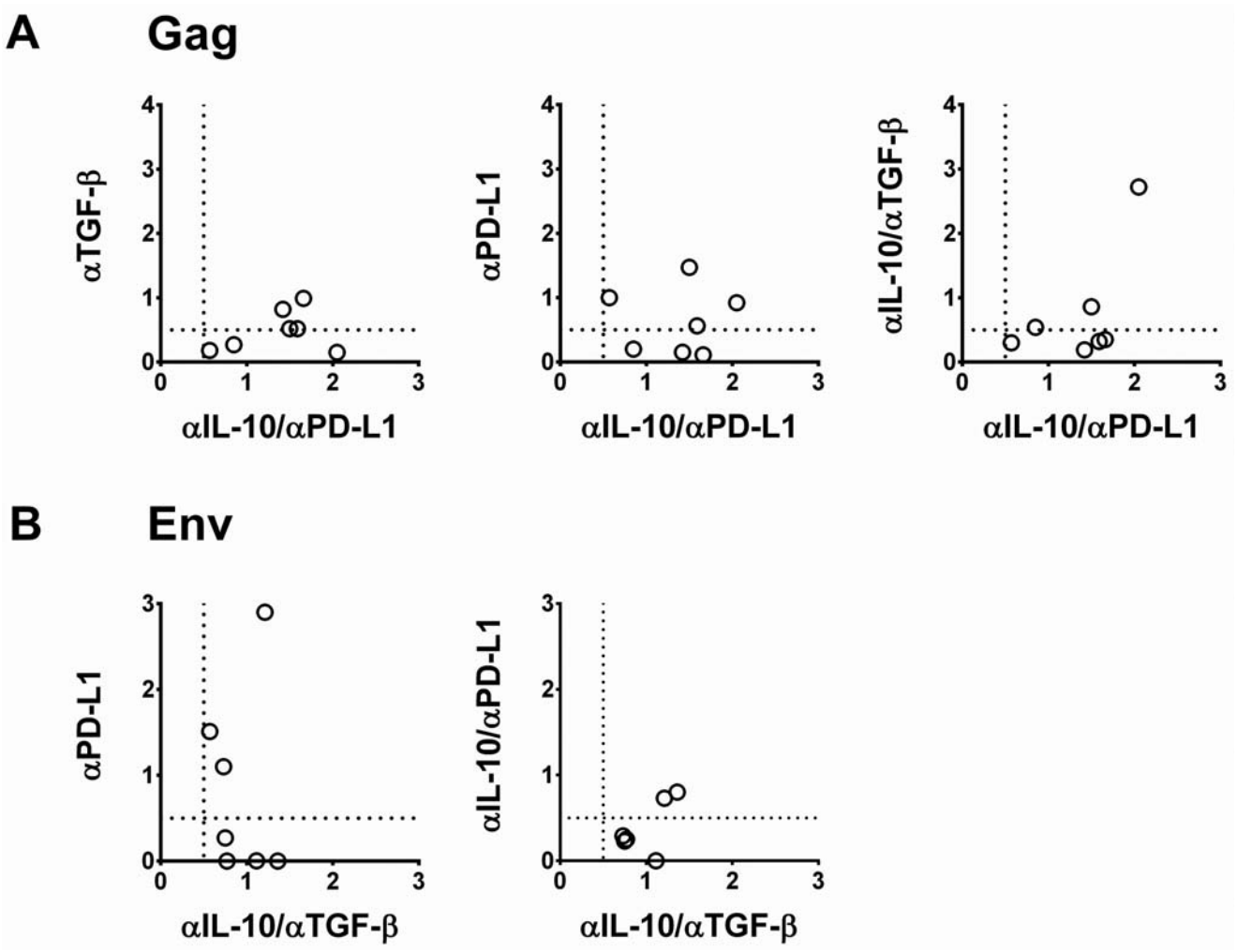

Fig 2. Gag and Env FTR is best identified by different combinations of blocking mAbs. The relationship between FTR mediated by various pathways in (A) Gag- and (B) Env-stimulated cultures. Plots include patients with detectable FTR on one or both axes. Only dual blockade of IL-10/PD-L1 (x-axis) identifies all patients with Gag FTR whereas dual blockade of IL-10/TGF-β (x-axis) identifies all patients with Env FTR. FTR defined as an increase in proliferating cells above a threshold of 0.5% (dashed lines).

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Env-regulators indeed had lower CD4 counts and higher HIV RNA than both Non- and Pan-regulators (see Fig 4).

Concordant *proliferative* responses to HIV Gag and Env

As observed in previous studies, the proliferative responses of $CD8^+$ T cells to Gag stimulation were greater than those induced by Env ($p = 0.046$, data not shown). In contrast to assessments of FTR, the $CD8^+$ proliferative responses to the two antigens correlated ($r = 0.65$, $p<0.001$). The $CD8^+$ T cell activation phenotype, as defined by co-expression of CD25 and HLA-DR, correlated closely with the proliferative responses in all culture conditions ($r = 0.76–0.89$, $p<0.001$).

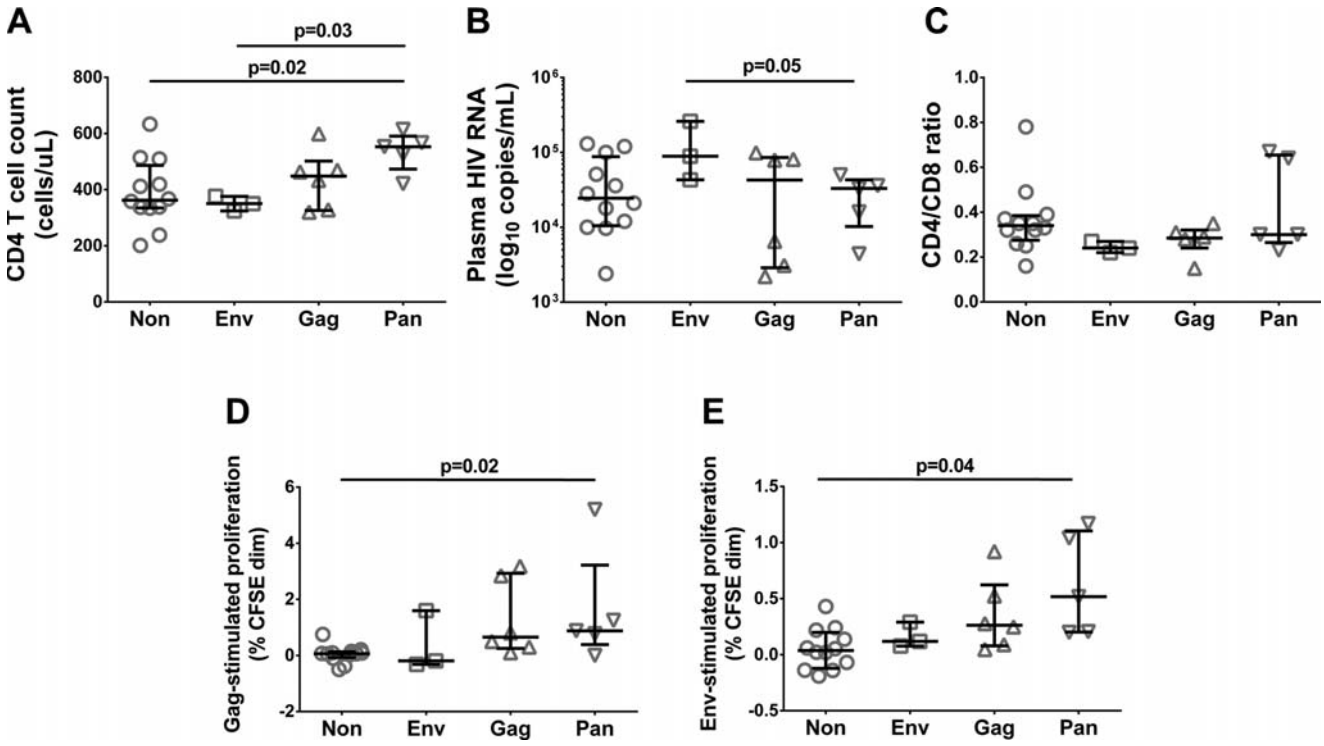

Fig 3. Clinical and immunological variables of regulator subgroups. (A) CD4 count, (B) Plasma HIV RNA, (C) CD4/CD8 ratio, (D) Gag-stimulated proliferation and (E) Env-stimulated proliferation, by regulator subgroup. Median and upper/lower quartiles indicated. P-values derived from unpaired tests.

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Underscoring the complex relationship between proliferation and regulatory mechanisms, no significant correlations were found between antigen-specific proliferative responses and FTR mediated by any inhibitory pathway.

Frequencies of activated Tregs following HIV antigen stimulation and signal blockade

In the study cohort as a whole, the percentage of aTregs increased significantly after both Gag and Env stimulation *in vitro* ($p<0.01$, Fig 5). Dual blockade of IL-10/PD-L1 in Gag-stimulated cultures increased the aTreg fraction further ($p = 0.007$), whereas no such increase were observed in Env-stimulated cultures with dual blockade of IL-10/TGF-β.

Discussion

The primary aim of this study was to explore and quantify antigen-specific functional T cell regulation by blocking defined inhibitory pathways in HIV antigen-stimulated cultures, with an ultimate goal of testing such parameters in the setting of functional HIV cure interventions. Our previous work to assess regulation with combined blockade of IL-10 and TGF-β was here expanded to include the PD-L1/PD-1 and CD160/HVEM pathways. We cross-sectionally tested samples from 26 HIV-infected, ART naïve patients, of who 14 (54%) had detectable FTR. Our main finding was substantial heterogeneity in the prevalence and magnitude of FTR between patients, inhibitory pathways as well as Gag and Env antigens, consistent with our previous observations [17]. Moreover, we have previously reported a similar variability in patients

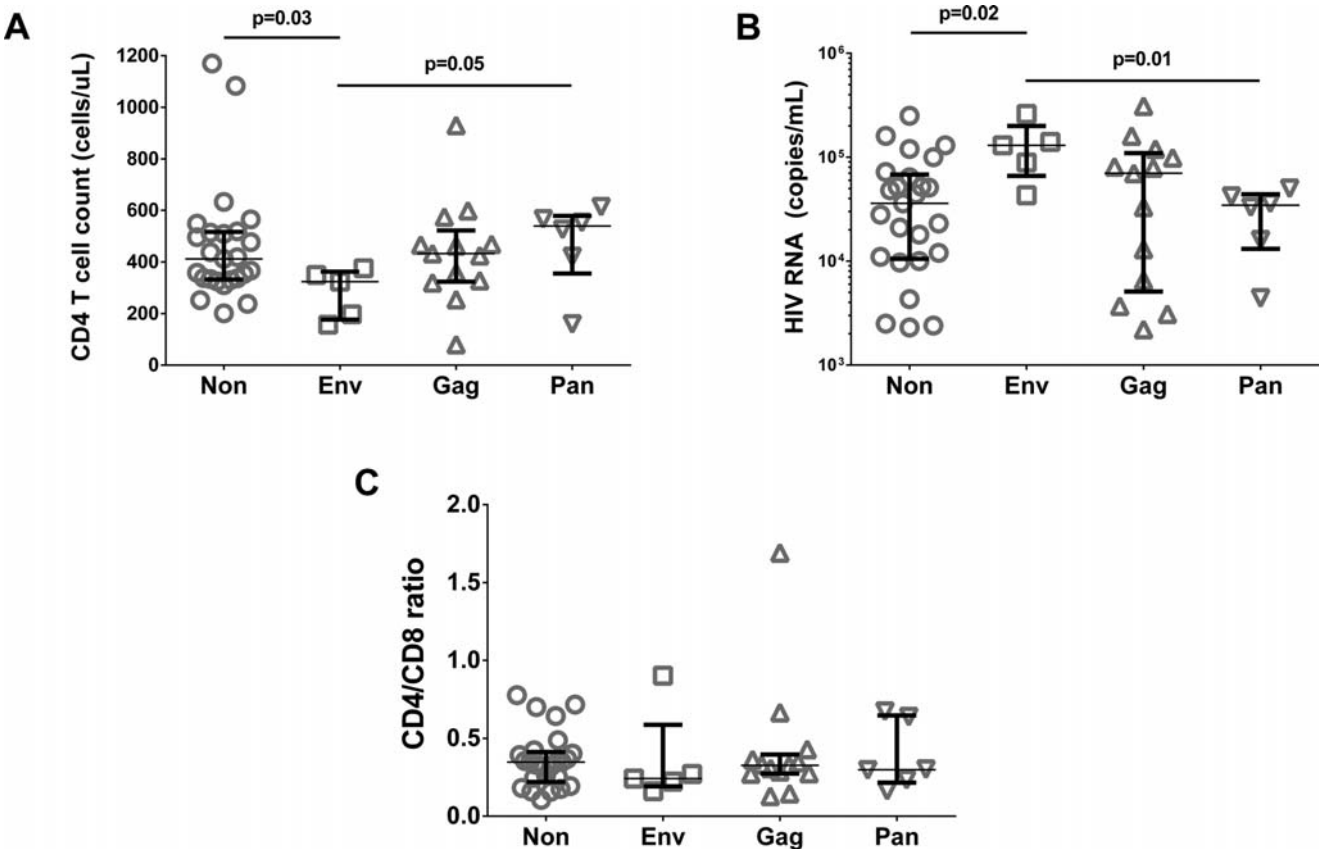

Fig 4. Lower CD4 counts and higher HIV viral loads in Env-regulators. (A) CD4 count, (B) Plasma HIV RNA and (C) CD4/CD8 ratio by regulator subgroup in the expanded cohort (n = 49). Median and upper/lower quartiles indicated. P-values derived from unpaired tests.

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on ART, where this type of assay may help select patients for and/or predict the efficacy of therapeutic HIV vaccines [19].

In preliminary experiments, Antigen-specific, Cytokine-mediated regulation (R_{AC}), as defined in our previous work [17] was also determined, taking into account downregulation of bystander proliferation in control cultures. We found minimal effects on background proliferation of blocking any of the assessed inhibitory pathways and thus a high concordance between RAC and FTR (data not shown).

We chose to use T cell proliferation after 5 days of culture as our primary read-out in assessing FTR. While interferon (IFN) γ secretion detected by ELISPOT or intracellular cytokine staining [25] and polyfunctionality [5,26] are commonly used measures of effector T cell responses to HIV peptides, these assays typically involve 6–18 hours of culture before analysis. We expected antigen-related regulation to develop more slowly, secondary to primary cell activation. In addition, the capacity of HIV-specific effector T cells to proliferate has been linked to delayed or non-progression of HIV infection [27,28], even at an epitope level [29]. Furthermore, proliferation has been linked to perforin expression by $CD8^+$ T cells [28], essential for cytolysis of infected target cells. The high concordance between T cell proliferation and expression of the activation markers CD25 and HLA-DR was here additional evidence of T cell activation processes involving proinflammatory cytokines such as IFN-γ and IL-2 [30,31].

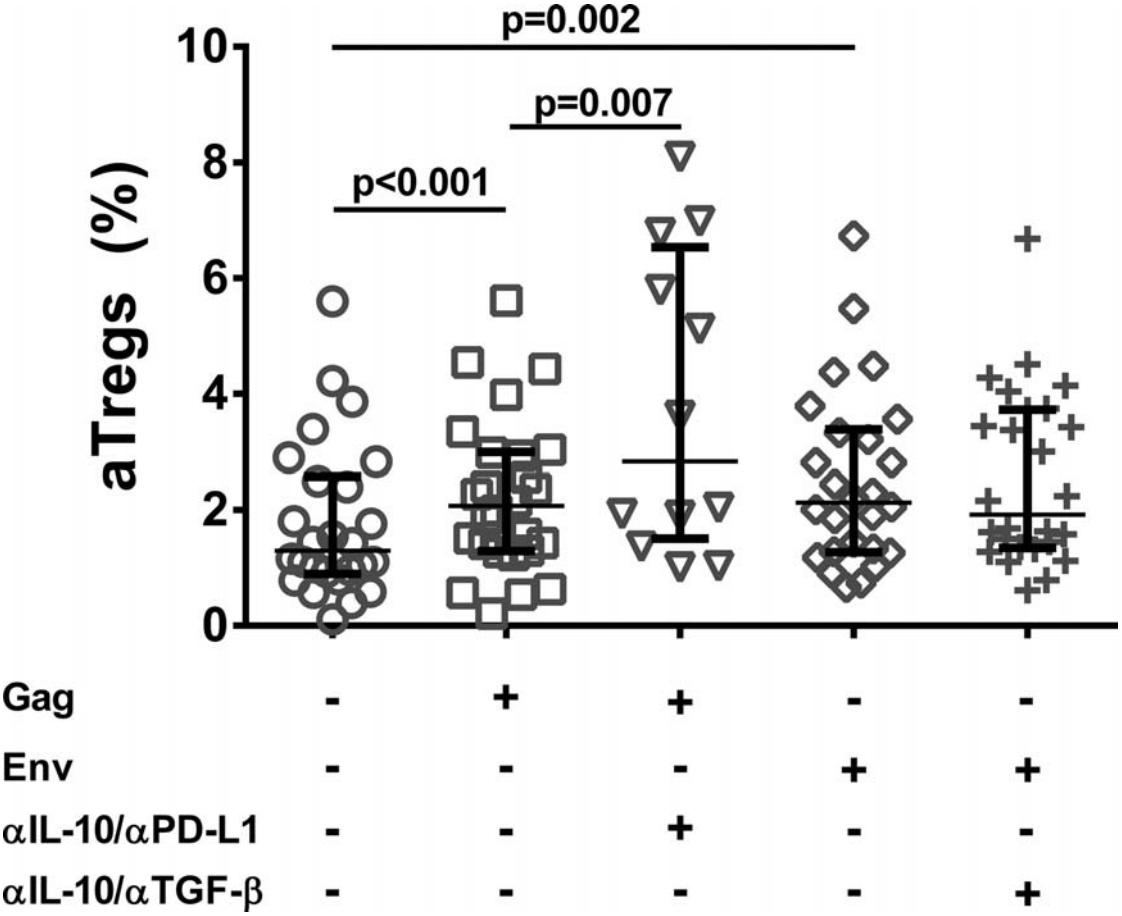

Fig 5. Increase in aTreg frequency after IL10/PD-1 dual blockade of Gag-stimulated cultures. Frequencies of activated regulatory T cells ($CD25^{hi}CD45RA^{-}$) in unstimulated cultures and in antigen-stimulated cultures with and without blocking antibodies. Median and upper/lower quartiles indicated. P-values derived from paired tests.

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In our previous studies, we have quantified functional regulation by dual blockade of IL-10 and TGF-β [17–19]. This assay condition still proved most effective in revealing Env FTR in the current study cohort. By contrast, Gag FTR was best identified by combined IL-10 and PD-L1 blockade, in keeping with Porichis et al. [23]. Blockade of the PD-1/PD-L1 pathway is known to restore cytokine secretion and proliferative capacity of exhausted $CD8^+$ T cells in HIV infection [32]. We found that the PD-1/PD-L1 pathway was involved in both Gag and Env FTR, but Gag-specific responses were more affected. As Gag-specific T cell responses in particular have been linked to control of HIV viremia [2,33], our data may lend further support to the use of anti-PD-1 antibodies in HIV immunotherapy [22]. Of note, the additive effect of concurrent PD-1 and IL-10 blockade was only apparent upon Gag stimulation.

Somewhat surprisingly, our assay neither detected Gag nor Env regulation by blocking IL-10 alone, as effects of IL-10Ra blockade on Gag p24-specific $CD8^+$ T cell proliferation have been reported [34]. Notably, the monoclonal antibody used in our study (clone 23738) neutralises IL-10 bio-activity [17,35]. On the other hand, TGF-β alone mediated regulation in some patients, in accordance with Garba et al. [36]. The additive effect of blocking IL-10 and TGF-β

in the same cultures may therefore suggest a potential recruitment of IL-10 dependent mechanisms during TGF-β blockade.

Regulatory effects of the CD160/HVEM pathway were detected in very few patients; two upon Gag stimulation and another patient upon Env stimulation. This contrasts with the results of Peretz et al. [37], who found significantly increased HIV-specific proliferation and cytokine secretion of $CD8^+$ T cells during HVEM blockade. However, their experiments utilized peptides from HIV antigens other than Gag and Env.

Non-regulator patients with little or no detectable FTR by any of the two HIV peptide pools were clinically heterogeneous, with a wide range of CD4 counts and viral loads. This group was, however, also characterised by weak antigen-specific proliferative responses (i.e. unblocked), an observation which was confirmed in the expanded cohort (data not shown). Whether other regulatory mechanisms may be at play, or whether these HIV-specific T cell clones have in fact been deleted cannot be determined by our experiments. In a recent trial of therapeutic vaccination in another HIV-positive cohort, however, we have observed that patients who would have been denominated Non-regulators at study baseline may still develop FTR against the vaccine antigens [unpublished data].

Regulator patients displayed the same clinical heterogeneity as Non-regulators. While the apparent paucity of Env-regulators (approx. 10% in the expanded cohort) limits the conclusions which can be drawn, one may speculate that this group, exhibiting low HIV-specific proliferative capacity of $CD8^+$ T cells, constitutes a patient phenotype more prone to clinical progression, in keeping with their higher HIV RNA levels compared to Non- and Pan-regulators. These findings are also in accordance with our previous work in another patient cohort, in which Env regulation was associated with accelerated CD4 loss [17]. Pan-regulators, meanwhile, had better T cell responses to both antigens, and induction of FTR may merely be a bystander effect of a strong proliferative drive in this group.

As could be expected, HIV antigen stimulation of PBMC increased the frequency of activated Tregs in culture. This potential for generation of Tregs after antigen exposure of $CD4^+$ T cells is well established [38]. Interestingly, we observed a further increase in aTreg frequencies in Gag-stimulated cultures with dual blockade of IL-10 and PD-L1, but not in Env-stimulated cultures with IL-10/TGF-β blockade. As both TGF-β and the PD-1/PD-L1 axis has been implicated in the induction of Tregs [39–42], the mechanisms contributing to these antigen-dependent differences are unclear, and cannot be elucidated by our straightforward *in vitro* assay.

The increase in ART coverage and potential shift of focus to functional cure strategies in HIV infection encourage the extension of our study to ART-treated patients with suppressed viremia. The limited number of patients in this study as well as the substantial heterogeneity in T cell proliferation, FTR and HIV antigen dependency restrict the conclusions we can draw. Furthermore, while the regulation profiles of $CD8^+$ T cells as described here may have clinical significance, a cross-sectional approach cannot ascertain whether they represent a cause or consequence of progression.

In conclusion, assuming that enhanced proliferation of $CD8^+$ T cells by blockade of key inhibitory pathways represents functional regulation *in vivo*, our data confirm that FTR of HIV-specific CTL responses is common in HIV-infected patients. Although our data indicate that Gag and Env antigens are differentially regulated in patients, we nevertheless found combinations of inhibitory pathway blockers that identified all patients with Gag and Env FTR, respectively. Taken together, our data support the notion that interventions to improve HIV-specific immunity should be individually tailored and that assessments of FTR should be further explored in clinical trials.

Supporting Information

S1 Fig.
(TIF)

Author Contributions

Conceived and designed the experiments: CP AL AMDR DK. Performed the experiments: CP. Analyzed the data: CP AL AMDR DK. Wrote the paper: CP AL AMDR DK.

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