Aspects of specific T cell activation and regulation in chronic HIV infection and after therapeutic immunisation

Thesis for the degree Philosophiae Doctor

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Mittagsruh

Über Bergen, Fluß und Talen,
Stiller Lust und tiefen Qualen,
Webet heimlich, schillert, Strahlen!
Sinnend ruht des Tags Gewühle
In der dunkelblauen Schwüle,
Und die ewigen Gefühle,
Was dir selber unbewußt,
Treten heimlich, groß und leise
Aus der Wirrung fester Gleise,
Aus der unbewachten Brust,
In die stillen, weiten Kreise.

Joseph von Eichendorff (1812)
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Oslo, February 2014
## 2 Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
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<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
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<tr>
<td>ART</td>
<td>(Highly active) antiretroviral treatment</td>
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<tr>
<td>CCR</td>
<td>Chemokine (C-C motif) receptor</td>
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<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>CFSE</td>
<td>Carboxyfluorescein succinimidyl ester</td>
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<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
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<td>CTLA</td>
<td>Cytotoxic T-Lymphocyte Antigen</td>
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<td>DC</td>
<td>Dendritic cell</td>
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<td>DTH</td>
<td>Delayed-type hypersensitivity skin tests</td>
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<td>FMO</td>
<td>Fluorescence minus one</td>
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<td>GALT</td>
<td>Gut-associated lymphoid tissue</td>
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<td>GM-CSF</td>
<td>Granulocyte macrophage colony-stimulating factor</td>
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<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
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<td>IL</td>
<td>Interleukin</td>
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<td>INF</td>
<td>Interferon</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<td>MALT</td>
<td>Mucosal-associated lymphoid tissue</td>
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<tr>
<td>mAbs</td>
<td>Monoclonal antibody</td>
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<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
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<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>PD</td>
<td>Programmed cell death protein</td>
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<td>PMT</td>
<td>Photo multiplying tubes</td>
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<tr>
<td>STI</td>
<td>Structured treatment interruption</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<td>TGF</td>
<td>Tumour growth factor</td>
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<td>Th</td>
<td>T helper cells</td>
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<tr>
<td>TIM</td>
<td>T-cell immunoglobulin domain and mucin domain</td>
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<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cells</td>
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3 Publications included

1. Intradermal vaccination of HIV-infected patients with short HIV Gag p24-like peptides induces CD4+ and CD8+ T cell responses lasting more than seven years.

Lind A, Sommerfelt M, Holmberg J O, Baksaas I, Sørensen B, Kvale D.


2. Boosters of a therapeutic HIV-1 vaccine induce divergent T cell responses related to regulatory mechanisms.


3. A parameter for IL-10 and TGF-β mediated regulation of HIV-1 specific T cell activation provides novel information and relates to progression markers.

Lind A, Brekke K, Pettersen F O, Mollnes T E, Trøseid M, Kvale D.

More than 30 years have passed since the advent of the acquired immunodeficiency syndrome (AIDS) pandemic. This sparked a perhaps unparalleled scientific effort to find its cause (Human immunodeficiency virus: HIV), to understand the immune pathogenesis and to develop a cure. Today we have a reasonable understanding of how the virus affects the immune system as well as effective antiretroviral treatment (ART) that has saved and improved millions of lives. Thanks to the rollout of ART in resource poor high-endemic areas, the worldwide HIV incidence rate is finally in decline for the first time since the beginning of the pandemic.

Nonetheless, the rate of new infection is still higher than the number of patients who start ART. Many patients are unaware of their infection and therefore start ART too late. ART is non-curative, demands lifelong adherence, causes side effects, induces viral resistance and does not eliminate the increased risk of non-AIDS events such as cancer and cardiovascular disease. A cure or a protective vaccine is not at hand, and there is a strong demand for new therapeutic options.

One such treatment option could be therapeutic vaccination. The aim of such vaccines is to improve anti-HIV responses in infected patients. Therapeutic vaccines may potentially decrease or halt the progression towards AIDS or even be an essential part of a future cure. However, none of the therapeutic vaccine candidates have achieved this in clinical trials this far. Many immune pathogenic aspects are still unexplored as is how immunisation influences quantitative and qualitative aspects of HIV immunity. There is no consensus on the optimal choice of antigen, delivery system or adjuvant. Most importantly, there is a lack of good immune correlates of efficacy that can be used when developing vaccines.
An essential immunological aspect that has been little explored in HIV is immune regulation. This mechanism exists to protect the host from damaging effects of immune responses, but probably also suppresses natural effective anti-HIV immunity. Immune regulation could also hamper the effect of therapeutic vaccination, or even be induced by the vaccine itself. Thus, a result of therapeutic vaccination could be the induction of HIV-specific immune regulation instead of improved anti-HIV responses.

The three papers included in this thesis examine T cell activation and regulation during chronic HIV and during reboost with a peptide based therapeutic vaccine candidate (Vacc-4x). The first paper describes long-term T cell vaccine memory in a formerly immunised cohort. The second paper describes the dynamics of T cell responses during and after Vacc-4x reboost including changes in T cell immune regulation. Finally, the third paper describes quantitative aspects of T cell regulation in natural untreated chronic HIV infection including a correlation between T cell regulation and disease progression.

To develop effective immune therapy, we still need a better understanding of immunological mechanisms for protection including further research of immune regulation in HIV. Therapy must be individualised to account for individual immunological differences in the host’s response to HIV and immunisation. Hopefully, this will give us effective treatment that will improve the perspectives for HIV infected patients worldwide.
5 Introduction

5.1 The origin and history of HIV

Human immunodeficiency virus-1 (HIV), the major cause of AIDS, originates from the Congo River area in central Africa, and was transfected from primates to humans during the late 19th or early 20th century (Keele, Van Heuverswyn et al. 2006, Worobey, Gemmel et al. 2008). The oldest known HIV infection was discovered in a preserved blood sample from a Congolese man taken in 1959 (Zhu, Korber et al. 1998), and the earliest retrospectively verified cases of AIDS were in a man from Missouri USA in 1969 and in a Norwegian sailor in 1976 (Froland, Jenum et al. 1988).

The HIV pandemic became apparent in 1981 when epidemiologists at the US Centers for Disease Control and Prevention noticed an abnormal clustering of Pneumocystis jiroveci pneumonia and Kaposi sarcoma among previously healthy young homosexual men in Los Angeles and New York (Gottlieb, Schroff et al. 1981). The same clustering was also seen in haemophiliac patients and in intravenous drug users. Common for all these patients was malaise, weight loss and rare opportunistic infections (Marx 1982). It was soon established that the cause had to be an infectious agent, transmitted through sexual contact or intravenous exposure to blood products leading to what today is termed the acquired immunodeficiency syndrome (AIDS).

HIV was isolated in 1983 (Barre-Sinoussi 1983), and soon after the virus was cloned and its genome sequenced (Wain-Hobson, Sonigo et al. 1985). A blood test was available from 1985, and the first active antiviral drug was licensed in 1987 (Fischl, Richman et al. 1987) followed by other drugs and steadily improving treatment options (Hammer, Squires et al. 1997, Palella, Delaney et al. 1998).
5.2 Epidemiology

The UNAIDS report on the Global AIDS Epidemics from 2012 estimated that 2.5 million people were infected with HIV in 2011, 19% fewer than 1999 and 21% fewer than in the peak year 1997 (http://www.unaids.org/globalreport). Additionally the number of AIDS-related deaths decreased from 2.1 million in 2004 to 1.7 million in 2011. This has mainly been attributed to the introduction and rolling out of effective ART, reducing both HIV related death and HIV transmission (Stanecki, Daher et al. 2010), but still there are approximately 7000 new infections per day. Consequently, the number of people living with HIV worldwide has risen from 29.4 million in 2001 to 34.0 million in 2011. Sub-Saharan Africa has the highest prevalence (median 4.9%), which accounts for 69% of all people living with HIV worldwide (Figure 1).

![Figure 1. Global prevalence of HIV in 2011 (http://www.unaids.org).](http://www.unaids.org)

In Norway, the number of patients living with HIV in 2012 was 5138 (The Norwegian Institute of Public Health, www.fhi.no). Of the 242 infected that year, 69% were males, 32% were men having sex with men, 69% had been
infected prior to immigration to Norway and 0.9% had been infected through intravenous drug abuse.

5.3 HIV-1 structure

HIV-1 (HIV) belongs to the lentiviruses, a subfamily of the human retroviruses, whose hallmark is the reverse transcription of RNA to DNA. The virion is 100 nm wide and has an icosahedral structure. It comprises of the external (gp120) and transmembrane part (gp41) of the envelope, the inner membrane (matrix) and the core capsid (p24). The core capsid contains genomic RNA, reverse transcriptase (p18), integrase and other enzymes needed for the replication cycle (Figure 2) (Foley, Apetrei et al. 2013).

The genome consists of three structural genes; gag encodes for core proteins like p24, pol encodes enzymes responsible for protease, reverse transcriptase and integration, and env encodes envelope glycoproteins (Figure 3). Six other genes (tat, rev, nef, vif, vpr and vpu) encode proteins responsible for host cell regulation, immune evasion and viral gene expression.
HIV is phylogenetically divided into four subtypes. Subtype M (“Major”) is the most virulent subtype and responsible for more than 90% of the infections worldwide. Subtypes N (“Non-M, Non-O”), O (“Outlier”) and P (“Pending the identification of further human cases”) have little or negligible global significance (Sharp and Hahn 2010).

Figure 3. HIV genome map (Adapted from www.hivbook.com).

5.4 Replication cycle

The replication cycle begins when HIV has infected a target cell either via the surface receptors CD4 in conjunction with CCR5 or CXCR4 found on CD4+ T helper cells or alternatively by direct cell-to-cell transfer (Figure 4). HIV RNA is transcripted into double-stranded proviral DNA by the viral enzyme reverse transcriptase and integrated into chromosomal hotspots in the host cell nucleus by the enzyme integrase. Depending on cell activation, the integrated proviral DNA is then transcripted and spliced to genomic RNA, which is translated and modified into viral proteins and enzymes. These are assembled to become a progeny virion within the cell’s endoplasmatic reticulum. Transcription and splicing is very inaccurate, causing high genotypic and phenotypic variability (Korber, Gaschen et al. 2001). Budding completes the replication cycle, where the virus receives its
outer membrane and external envelope. During this process the virus can incorporate host cell proteins such as HLA class molecules, which further facilitates the infection of new target cells (Capobianchi 1996).

![Diagram of HIV Replication Cycle](Image)

**Figure 4. HIV Replication Cycle (National Institute of Allergy and Infectious Diseases, www.niaid.nih.gov).**

### 5.5 Clinical manifestation of untreated HIV infection

In untreated HIV infected patients, the median time from primary infection to the occurrence of AIDS-defining illnesses is 10 years, but this can range from less than a year to decades depending on various viral and host factors (Levy 2009). The median time from the development of AIDS-defining illnesses to death is two years. The clinical symptoms are usually divided
into three stages (Poli, Pantaleo et al. 1993) (Figure 5). Three to six weeks after primary infection, 50-70\% of the patients develop mononucleosis-like symptoms with lymphadenopathy, pharyngitis, malaise, myalgia, rash and fever. These symptoms vary and are frequently neglected or misjudged. Within the acute stage, the viral load reaches extreme values, and the CD4+ T cell numbers plunge considerably (Little, McLean et al. 1999, Fiebig, Wright et al. 2003). Symptoms mostly subside when virologic control has been established after approximately 12 weeks, but many patients experience fatigue, frequent herpes zoster outbreaks, skin conditions and lymphadenopathy. During the chronic stage, the viral load declines to reach an individual viral setpoint and the CD4+ T cell numbers recover incompletely (Mellors, Rinaldo et al. 1996). Still there is continuing viral replication and a relentless CD4+ T cell infection and loss. During the end-stage, viral control is lost due to a critical reduction in CD4+ T cell numbers combined with a precipitous decline in CD8+ cytotoxic T cells. This stage is dominated by constitutional symptoms and symptoms from a wide spectrum of opportunistic infections and neoplasms, to which the untreated patient finally will succumb (Lewin-Smith, Klassen et al. 1998).

Figure 5. The disease development from primary HIV infection until the onset of opportunistic infection and death (Figure adapted from Pantaleo, Graziosi et al. 1993).
5.6 **Pathogenesis**

HIV leads to progressive immunodeficiency from early and on-going depletion of T helper cells and from general chronic immune activation. Pathophysiological event within days after primary infection determines the course of the disease (Douek, Roederer et al. 2009).

5.6.1 **Transmission and early infection**

Transmission predominantly results from sexual contact but can also occur directly through contaminated blood products or during pregnancy, birth and breastfeeding (Pope and Haase 2003, Cohen, Shaw et al. 2011). HIV crosses damaged mucosa as virions or within infected cells, or can even overcome intact mucosa bond to receptors on dendritic cells (DC) (Figure 6). Several factors influence infectiveness, these include viral fitness, host genetics and the efficacy of preformed or induced host immune responses. During the first few days, viral replication is low and limited to locally infected CD4+ T cells (Douek, Brenchley et al. 2002). Soon the virus disseminates to lymphatic tissue that remains the main site of viral replication also during later stages. The gut-associated lymphoid tissue (GALT) is preferably infected as it contains high numbers of activated CD4+CCR5+ T cells. This includes 70-80% of all CD4+ memory T cells and most Th17 cells (Brenchley, Schacker et al. 2004, McMichael, Borrow et al. 2010). Th17 cells are a subset of IL-17 producing CD4+CCR5+ T cells who are responsible for mucosal viral and fungal defence and probably important for the maintenance of gut integrity (Hunt 2010).

CD4+ T cells are massively depleted during primary infection, including more than 50% of the GALT-associated CD4+ T cells (Mehandru, Poles et al. 2007). Infected cells succumb to the cytotoxic effects of viral replication or die from apoptosis or killing by HIV-specific cytotoxic T cells (CTL). Large numbers of uninfected CD4+ T cells are depleted due to erroneous
immune responses or activation-associated apoptosis (so-called bystander depletion) (Finkel, Tudor-Williams et al. 1995). These events are accompanied by unsustained viral replication, dissemination to still non-infected lymphoid tissue, and the establishment of latent viral reservoirs in resting CD4+ T cells and macrophages (Daar, Moudgil et al. 1991). Latent viral reservoirs are insensible to current ART and major obstacles for HIV eradication (Chun, Finzi et al. 1995, Siliciano, Kajdas et al. 2003, Richman, Margolis et al. 2009).

Figure 6. Mucosal transmission and infection of CD4+ T cells by HIV (Figure adapted from Moir, Chun et al. 2011).

5.6.2 Chronic immune activation

Generalised immune activation is a hallmark of HIV infection and has been identified as the main driver of disease progression towards AIDS (Appay and Sauce 2008, Moir, Chun et al. 2011, Hunt 2012). Additionally, immune activation probably contributes to accelerated aging and non-AIDS related disease such as bone fragility, cancers, cardiovascular disease, kidney disease and neurocognitive dysfunction, and is not completely normalised by current ART regimens in many patients (Deeks and Phillips 2009).

Some degree of immune activation is normal during any form of foreign antigen encounter. Once the antigen has been cleared, the immune system returns to an inactivated state with a pool of resting antigen-specific
memory cells. In chronic HIV infection however, the pathogen is not cleared, and this leads to an increased production of proinflammatory factors (McMichael, Borrow et al. 2010). Furthermore, there is a systemic influx of highly immunogenic bacterial products including lipopolysaccharides (LPS) from the gut lumen, possibly due to early and extensive virus-induced death of MALT-associated Th17 T cells with secondary dysfunction of tight junctions (Brenchley, Price et al. 2006, Sandler and Douek 2012). Adding to this are multiple other interacting elements not completely understood but under intense investigation. Some of these may be coinfections, residual viral replication, bystander apoptosis, depletion of T regulatory cells and dysregulation of innate immune responses (Garg, Mohl et al. 2012).

The pathogenic consequence is enhanced compensatory T cell proliferation combined with immune exhaustion and premature apoptosis leading to a gradual loss of the T cell pool (Grossman, Meier-Schellersheim et al. 2006) (Figure 7). This further impairs the ability of the immune system to mount effective antiviral responses. Immune activation sustains HIV infection and replication, as the virus thrives on activated CD4+ T cells, which in turn drives immune activation. Chronically activated T cells lose their capacity to proliferate, concurrently produce various cytokines and kill target cells. Activation also induces the upregulation of negative immune regulatory mechanisms, probably further hampering effective T cell responses (see chapter 5.7).
Figure 7. A vicious circle driven by HIV replication, T cell activation and the influx of bacterial products from the gut lumen sustains immune activation. This in turn drives HIV replication with the generation of HIV escape mutants, increases T cell activation, susceptibility, exhaustion and apoptosis, and upregulates immune regulatory mechanisms with increased HIV tolerance. The result is a progressive loss of immune control (Figure by D. Kvale, unpublished)

5.6.3 HIV related T cell dysfunction

CD4+ T cells are central conductors of the adaptive immune system, and collaborate with B cells and CD8+ T cells to prevent or clear infection (Castellino and Germain 2006). CD4+ T cells are activated by dendritic cells via HLA II, and can themselves activate CD8+ T cells via HLA I. CD8+ T cells can for example not enter mucosal tissue effectively without CD4+ T cell help (Nakanishi, Lu et al. 2009). Functional CD4+ T cells are therefore essential for the maintenance of effective CD8+ T cell responses.

By targeting CD4+ T cells, the virus critically hampers the capability to clear viral infection. Particularly HIV-specific CD4+ T cells are infected and eliminated early on as they are highly activated and home to infected lymphatic sites (Douek, Brenchley et al. 2002). Still, most CD4+ T cells remain uninfected in chronic HIV, but are either subjected to bystander depletion or gradually become exhausted and lose effector functions such as the ability to secrete IL-2 (Younes, Yassine-Diab et al. 2003). CD8+ T cells are also progressively exhausted and prematurely die under the strain
of chronic immune activation, ongoing antigen pressure (Bucks, Norton et al. 2009), various inhibiting factors, and insufficient CD4+ T cell help (Matloubian, Concepcion et al. 1994, Wherry and Ahmed 2004, Jin, Jeong et al. 2011, Wherry 2011) (Figure 8). This also preferably occurs to HIV-specific clones, further narrowing down the ability of the immune system to react to new viral escape mutants. Chronic immune activation also leads to immune senescence and accelerated cell aging due to telomere erosion and unrepaired DNA damage.

![Figure 8. During chronic HIV, CD8+ T cells progressively and hierarchically lose effector functions starting with the proliferative and killing capacity and ending with the cessation of INF-γ production and apoptosis (Rosenberg, Billingsley et al. 1997, Appay, Nixon et al. 2000, Migueles, Laborico et al. 2002, Betts, Nason et al. 2006). This progressive T cell exhaustion is driven by factors such as loss of CD4+ T cell help, high antigen/T cell ratio (Streeck, Brumme et al. 2008), dysfunctional or suppressive antigen presenting cells, and an increase in inhibitory molecules and immune suppressive cytokines (Figure adapted from Wherry and Ahmed 2004).]

5.6.4 Viral escape

HIV mutates quickly due to the inaccurate reverse transcriptase, which leads to the formation of a high number of mutant variants especially during high viraemia. Mutation enables the virus to escape effective immune control, but can also result in a loss of viral fitness. Sequence analyses of mucosally transmitted HIV have suggested that infection occurs by only one or a few homogenic founder virus, but that these quickly mutate under immunologic pressure (Keele, Giorgi et al. 2008). Furthermore, HIV possesses various mechanisms such as HLA class I downregulation or the glycosylation and masking of important viral
epitopes, which further aggravates the chances of immune clearance (Capobianchi 1996).

### 5.7 Regulation of T cell responses

A vital feature of the immune system is the delicate balance between positive (activating) and negative (regulating) signals that ensure adequate immune responses without damaging the host (Kaufmann and Walker 2009). An imbalance between these signals may lead to inappropriate immune reactions with potentially lethal consequences or conversely to suppressed immune clearance and chronification of the infection (De Keersmaecker, Allard et al. 2012).

Also T cells are strictly controlled by a complex system of activating and inhibitory factors and cells that is not yet fully understood (Fahey and Brooks 2010, Bour-Jordan, Esensten et al. 2011) (Figure 9). The final T cell response is only the net result of activation and regulation where regulatory factors actively restricts T cell effector functions such as activation, proliferation and degranulation (Wherry, Ha et al. 2007, Appay, Douek et al. 2008).

T cell regulation may be mediated by immune cells such as regulatory T cells (Treg) (Rouse, Sarangi et al. 2006, Belkaid and Tarbell 2009), DC (Probst, McCoy et al. 2005) and suppressor CD8+ T cells (Elrefaei 2008, Elrefaei, Burke et al. 2009), by increased expression of inhibitory receptors such as cytotoxic T-lymphocyte antigen-4 (CTLA-4) (Kaufmann, Kavanagh et al. 2007) and programmed cell death molecule 1 (PD-1) (Keir, Butte et al. 2008), and by soluble inhibitory cytokines such as interleukin-10 (IL-10) and transforming growth factor-β (TGF-β) (Letterio and Roberts 1997, Couper, Blount et al. 2008, Wilson and Brooks 2011). These factors inhibit T cell responses simultaneously and relate to T cell exhaustion and loss of T cell activity (Blackburn, Shin et al. 2009). Several of these regulatory pathways are altered in chronic HIV-infection and have
shown to correlate unfavourably with proliferative capacity, viral load and CD4 counts (Kaufmann and Walker 2009, Kassu, Marcus et al. 2010). Still, whether these alterations are induced by the virus or are secondary to immune activation is not clear, nor is how these alterations individually and collectively influence HIV immunity during different stages of the disease (Fahey and Brooks 2010).
Figure 9. The role of immune regulation during chronic viral infection. A delicate balance between positive (green) and negative (red) regulatory signals ensures adequate immune responses without damage to the host (a). Alterations can either tip the scales toward increased T cell responses that could clear chronic infection (b), or negative regulation with increased T cell exhaustion and viral persistence (c). Therapeutically blocking negative pathways, for example by using anti-PD-1, anti-IL10 or anti-TGF-β monoclonal antibodies as suggested in (b), enhances T cell activity in vitro. In vivo this may improve immune control of chronic viral infections, but with the danger of serious autoimmune reactions (Figure adapted from Fahey and Brooks 2010).
5.7.1 Inhibitory CD4+ T cells

T regulatory cells (Treg) are a subset of CD4+ T cells important for self-tolerance and T cell regulation (Belkaid and Tarbell 2009). They constitute approximately 5-10% of all peripheral CD4+ cells and are (mostly) defined by the high expression of the IL-2 receptor (CD25), low expression of the IL-7 receptor (CD127) and the expression of the transcription factor forkhead box protein 3 (FOXP3) along with other phenotypic markers (Read, Malmstrom et al. 2000, Hori, Nomura et al. 2003).

Treg elicit their effect on T cells and DC via cell-to-cell contact, cytolysis or via the inhibitory cytokines IL-10 and TGF-β (Figure 10). Natural Treg (nTreg) are generated in the thymus from early on and play a pivotal role in preventing autoimmunity. Peripherally induced Treg (iTreg) develop from naïve T cells upon antigen stimulation and regulate T cell responses against foreign antigens. Treg are also found in high numbers in the gut where they are reciprocally related to Th17 cells and where they induce beneficial tolerance for food allergens and commensal bacteria (Prioult and Nagler-Anderson 2005). Apart from peripheral tolerance, Treg have been shown to promote the establishment of chronic viral, parasitic and fungal infections and to suppress anti-tumour responses (Liu, Wong et al. 2007, Belkaid and Tarbell 2009).

In HIV infection, the absolute and relative Treg numbers seem to increase during the chronic stages, but how this influences disease progression is still not clear. (Hunt, Landay et al. 2011, Presicce, Orsborn et al. 2011, Moreno-Fernandez, Presicce et al. 2012). Treg, like immune regulation in general, may in fact be a double-edged sword having both positive and detrimental effects on HIV-related immunity depending on the stage of the disease (Fazekas de St 2008, Chevalier and Weiss 2013). In acute HIV, Treg may reduce immune activation and indirectly the number of activated T cells, and thus reduce available targets for HIV (Kinter, Hennessey et al.
In chronic HIV, Treg may suppress effective polyfunctional antiviral responses and thereby increase disease progression (Aandahl, Michaelsson et al. 2004, Andersson, Boasso et al. 2005, Montes, Lewis et al. 2006, Nilsson, Boasso et al. 2006). More recent data have suggested that low Treg responses are associated with viral control in HIV controllers and that protective HIV-specific CD8+ T cells evade Treg suppression (Elahi, Dinges et al. 2011, Hunt, Landay et al. 2011). Additionally, Treg may initiate immune dysfunction by massive TGF-β secretion, which induces lymph node fibrosis. This leads to impaired maintenance of the T cell population as well as disrupted T cell activation (Pal and Schnapp 2004, Estes, Wietgrefe et al. 2007).

**Figure 10. Principle modes of Treg elicited immune regulation.** The production of inhibitory cytokines (a), cytolysis of effector T cells (b), metabolic disruption including IL-2 deprivation (c), and the inhibition of dendritic cell maturation (d) (Figure adapted from Vignali, Collison et al. 2008).
5.7.2 Co-inhibitory molecules

Co-inhibitory molecules constitute negative feedback mechanisms and may induce reversible or irreversible T cell inhibition, dysfunction and apoptosis. In chronic viral infection it has been shown that exhausted CD8+ T cells express up to seven different inhibitory receptors and that the co-expression of these receptors are associated with T cell exhaustion and the inability to control infection. These molecules are also upregulated in chronic HIV infection and coincide with T cell exhaustion (Kaufmann and Walker 2008, Blackburn, Shin et al. 2009).

One example is the upregulation of PD-1, which induces a strong inhibitory signal on effector T cells. PD-1 interacts with its ligand on antigen presenting cells (APC), contributing to decreased T cell receptor (TCR) functions and diminishing T cell responses (Crawford and Wherry 2009, Kaufmann and Walker 2009, Pettersen, Tasken et al. 2010, Porichis and Kaufmann 2011). Other upregulated inhibitory molecules in chronic HIV are CTLA-4 on CD4+ T cells and T-cell immunoglobulin domain and mucin domain 3 (TIM-3) on cytotoxic CD8+ T cells (Leng, Bentwich et al. 2002, Anderson and Anderson 2006, Khaitan and Unutmaz 2011).

It has been shown that blockade of these inhibitory receptors have restored T cell functions. Co-inhibitory molecules have therefore been suggested as potential targets for immune modulatory therapies (Day, Kaufmann et al. 2006, Blackburn, Shin et al. 2009, Simone, Piatti et al. 2009, Porichis and Kaufmann 2012, Wolchok, Kluger et al. 2013).

5.7.3 T cell Inhibitory cytokines and chemokines

Cytokines and chemokines are not only important for activating and directing T cell responses, but also have a central role in immune regulation. The most prominent T cell inhibiting cytokines are IL-10 and TGF-β, and both have been associated with HIV-disease progression
IL-10 is mainly produced by monocytes, but is also secreted by lymphocytes such as CD8+ T cells, Treg and B cells (Couper, Blount et al. 2008, Saraiva and O'Garra 2010). It is pleiotropic as it deters the expression of Th1 cytokines and the functions of APC, but enhances B cell proliferation and antibody production. Studies on IL-10 depleted knockout mice have shown the development of inflammatory bowel disease and immune overreaction during immune challenges. Furthermore, IL-10 has been associated with T cell exhaustion and the establishment of chronic infection (Clerici, Wynn et al. 1994). In chronic HIV infection, elevated IL-10 levels have been correlated with disease progression (Clerici, Wynn et al. 1994, Stylianou, Aukrust et al. 1999). IL-10 blockade has enhanced proliferative T cell responses to Env peptides and increased INF-γ and IL-2 secretion (Brooks, Lee et al. 2008, Brockman, Kwon et al. 2009). Another study associated IL-10 production to PD-1 expression on monocytes, which in turn was triggered by microbial translocation (Said, Dupuy et al. 2010).

TGF-β is also pleiotropic as it, among others, inhibits T cell proliferation and differentiation, but promotes the survival of peripheral T cells (Letterio and Roberts 1998, Li and Flavell 2008). TGF-β has been associated with CTLA-4 upregulation and the development lymph node fibrosis (Pal and Schnapp 2004, Elrefaei, Burke et al. 2009).
5.8 HIV treatment

5.8.1 The effects and limitations of ART

ART has revolutionised patient treatment and dramatically reduced HIV morbidity and mortality (Palella, Delaney et al. 1998). ART increases the number of CD4+ T cells, improves memory T cell responses, and decreases immune activation (Autran, Carcelain et al. 1997). Indirectly, ART also prevents new infection, because a reduced viral load lowers the risk of HIV transmission (Cohen, Smith et al. 2013). Still, ART is non-curative since it is not able to eliminate latent HIV reservoirs and it does not completely suppress viral replication and cell-to-cell infection (Furtado, Callaway et al. 1999, Chun, Nickle et al. 2005, Rong and Perelson 2009). HIV-specific CD4+ T cells do not recuperate during ART, and HIV-specific CTL responses diminish due to low HIV antigen exposure (Kalams, Goulder et al. 1999, Carr and Cooper 2000, Nachega, Marconi et al. 2011). HIV replication rebounds quickly when treatment is stopped, even in patients who start ART early (Davey, Bhat et al. 1999, Kvale, Kran et al. 2005). ART does not eradicate latent viral reservoirs and there is still a risk of developing viral resistance (Barton, Burch et al. 2013, Beyrer and Abdool Karim 2013). In addition, ART demands life long adherence, potentially causes side effects, and does not eliminate the increased risk of non-AIDS events such as cancer and cardiovascular disease (Richman, Margolis et al. 2009, Desai and Landay 2010). Last but not least, ART is not available for a majority of the patients in the developing world where the HIV epidemic still causes great human, social and economic harm.

5.8.2 Other possible treatment options

As ART neither cures the patient nor stops the disease completely, there is a need for alternative treatment options. The ultimate goal is eradication, a goal that has not been reached after more than 30 years of HIV research.
The only (probably) cured patient this far is the “Berlin patient” who received a bone marrow transplant with CCR5 receptor mutated stem cells, which effectively stopped the virus from entering new uninfected cells (Hutter, Nowak et al. 2009, Allers, Hutter et al. 2011). Also possibly cured was an infant infected with HIV during birth and who received ART within 30 hours. The infant became virus-free and remained so even after discontinuation of the treatment (Persaud, Gay et al. 2013). Other eradication approaches such as gene therapy, stem cell treatment, intensified ART, and reactivation strategies are under investigation, but with no positive results so far (Chun, Stuyver et al. 1997, Finzi, Hermankova et al. 1997, Garcia, Leon et al. 2012, Vanham and Van Gulck 2012).

5.8.3 Prophylactic HIV vaccines

Vaccines are the safest, cheapest and most effective way of controlling infection in large populations. Thus, a prophylactic HIV vaccine has been a main goal from the beginning of the endemic.

The aim of vaccination is to trigger a broad spectre of antigen-specific B and/or T memory responses. Several efficacy trials on prophylactic vaccines have been conducted since the discovery of HIV, but with disappointing results (Haynes, Liao et al. 2010, Kim, Rerks-Ngarm et al. 2010). The VAX003 and VAX004 studies in 2003 aimed at inducing broadly neutralising antibodies (Pitisuttithum, Gilbert et al. 2006, Jones, DeCamp et al. 2009). However, these antibodies did not prevent viral entry, either because they bound to structurally irrelevant epitopes or due to viral mutation or masking of important epitopes. The STEP trial (HVTN 502) in 2007 aimed at inducing effective T cell response. It had to be stopped due to non-efficacy and a higher infection risk in immunised patients with preformed antibodies against the vaccine adjuvant Ad5 (Buchbinder, Mehrotra et al. 2008). The RV144 trial in 2009, which aimed at inducing
both B and T cell responses, showed a moderate (31%), but only temporary protective effect (Rerks-Ngarm, Pitisuttithum et al. 2009). Finally, the most recent clinical vaccine trial (DNA/rAd5) was stopped in 2013 due to lack of efficacy (Hammer, Sobieszczyk et al. 2013).

New optimism can be drawn from the astonishing study results on a CMV vectored simian immunodeficiency virus (SIV) vaccine. Fifty per cent of the immunised and repeatedly virally challenged rhesus macaques showed durable aviraemic control even after demonstrable lymphatic and haematogenous dissemination, and regardless of the route of infection (Hansen, Piatak et al. 2013). Whether these results can be reproduced or translated into human prophylactic HIV vaccines remains to be seen.

5.8.4 Therapeutic HIV vaccines

Therapeutic HIV vaccines are intended to improve HIV-specific immunity in infected patients using viral or virus-like antigens (Garcia, Leon et al. 2012). Ideally, vaccine-induced responses would control the infection and halt progression to AIDS without additional treatment (“functional cure”), or at least partially suppress replication and delay the initiation of ART (Deeks, Autran et al. 2012). Another possible aim may be to induce responses that could help eliminate resting HIV reservoirs in combination with other treatment.

Initially, therapeutic HIV vaccines aimed at saving ART, since treatment was costly, cumbersome, and associated with serious side effects and development of viral resistance. Improved HIV-specific immune responses were supposed to postpone treatment or enable the patient to stop ART. However, due to an improved and simplified treatment regimen, and as a consequence of the SMART study, the consensus now is to start ART earlier and to refrain from treatment interruptions (Lundgren, Babiker et al. 2008). Recent reanalysis of the SMART study data indicate that treatment interruption can be safe for some patient categories (Paton 2008, Routy,
Boulassel et al. 2012). Still, the current view is that therapeutic vaccines will have to be a supplement rather than an alternative for other treatment.

Numerous therapeutic vaccine candidates have been tested in human trials (Reviewed in Haynes, Liao et al. 2010). Early therapeutic vaccines comprised of whole inactivated virus or recombinant viral peptides such as recombinant gp120 to stimulate the generation of neutralising antibodies. Later approaches have been DNA-based vaccines, the use of viral vectors, and peptides or dendritic cell-based vaccines (Reviewed in Garcia, Leon et al. 2012). Few if any of these vaccines have had positive and lasting effects on “hard” clinical endpoints such as viral load, CD4 T cell counts, or disease progression, perhaps with a few exceptions (see Table 1).
### Table 1. Selected human therapeutic vaccine trials

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Vaccine type</th>
<th>Possible clinical benefits</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vacc-4x</strong> (Kran, Sorensen et al. 2004, Kran, Sommerfelt et al. 2005, Kran, Sorensen et al. 2006, Kran, Jonassen et al. 2010).</td>
<td>Subunit vaccine containing Gag concensus peptides (see details below).</td>
<td>Reduced viral loads and delayed reintroduction of ART after structured treatment interruption (STI) with no observed immune escape.</td>
</tr>
<tr>
<td><strong>DermaVir</strong> (Lisziewicz, Trocio et al. 2005, Gudmundsdotter, Wahren et al. 2011).</td>
<td>Topical plasmid DNA vaccine expressing 15 different HIV-1 antigens.</td>
<td>Reduced viral load after STI.</td>
</tr>
<tr>
<td><strong>TAT</strong> (Ensoli, Bellino et al. 2010).</td>
<td>Whole Tat protein.</td>
<td>Not assessed. Decreased immune activation.</td>
</tr>
<tr>
<td><strong>AIDS Clinical Trials Group 5197</strong> (Schooley, Spritzler et al. 2010).</td>
<td>Recombinant adenovirus expressing HIV-1 Gag.</td>
<td>Control of viral replication associated with vaccine-specific CD4+ T cells.</td>
</tr>
<tr>
<td><strong>CAF01</strong> (Karlsson, Brandt et al. 2013).</td>
<td>18 subdominant HIV peptides in conjunction with a cationic adjuvant (CAF01)</td>
<td>Not proven.</td>
</tr>
<tr>
<td><strong>DCV2/MANON07-ORVACS Study Group</strong> (Garcia, Climent et al. 2013).</td>
<td>Autologous DC pulsed with whole inactivated HIV-1.</td>
<td>Decreased viral load during STI.</td>
</tr>
<tr>
<td><strong>Vacc-4x Multicentre Study</strong> (Pollard, Rockstroh et al. 2014).</td>
<td>Double-blind, randomised, phase 2 study comparing Vacc-4x to placebo. Reboost study planned.</td>
<td>Reduction in viral load relative to pre-ART after STI in Vacc-4x immunised patients compared to placebo group.</td>
</tr>
</tbody>
</table>

*Evaluation of immunised HIV-infected patients from the RV144 trial.*
5.8.5 Inducing effective immunity against HIV

Current therapeutic vaccine candidates mostly aim at inducing broadly reacting CD8+ T effector cells (CTL), since these cells are able to eliminate virus-infected cells.

For once, it has been shown that HIV-specific CD8+ T cells are able to control viraemia in acute HIV, and that some of these responses induce viral escape mutations (Appay and Sauce 2008, McMichael, Borrow et al. 2010, Moir, Chun et al. 2011). The presence of certain HLA I type alleles has been associated with improved viral control, possibly due to superior cross-reactivity (Saah, Hoover et al. 1998). While there is no correlation between the frequency of HIV-specific T cells and viral load, the quality of T cell response seems to be crucial (Rosenberg, Billingsley et al. 1997, Betts, Ambrozak et al. 2001). The ability of CD8+ T cells to proliferate, simultaneously produce various cytokines (particularly IL-2, TNF-a and IFN-γ), and rapidly secrete perforins and Granzyme B has been correlated to slower disease progression (Migueles, Laborico et al. 2002, Zimmerli, Harari et al. 2005, Betts, Nason et al. 2006, Hersperger, Migueles et al. 2011). Such responses are found in so-called elite controllers who are HIV-infected patients with suppressed viraemia and maintained high CD4 counts for years without treatment (Deeks and Walker 2007, Autran, Descours et al. 2011). Another important factor is epitope specificity. Patients with strong responses to Gag epitopes have slower progression towards AIDS than patients with strong Env responses (Edwards, Bansal et al. 2002, Kiepiela, Ngumbela et al. 2007, Pettersen, Tasken et al. 2010). A possible explanation for this may be that Env more readily mutates under immune pressure than Gag, and that this happens without fitness cost for the virus. Additional properties that have been connected to superior control are the clonal breadth, the procession of public clonotypes, and high antigen avidity (Iglesias, Almeida et al. 2011).
In conclusion, for a therapeutic vaccine to be effective, it should be able to induce strong proliferative, polyfunctional and cytotoxic HIV-specific T cells including durable T cell memory. In order to have a clinical impact, such T cells should theoretically be directed against conserved Gag epitopes and latent infected cells.

5.9 A short introduction to the previous Vacc-4x immunisation study

Subunit vaccines are peptides or recombinant HIV proteins designed to induce strong and polyfunctional cytotoxic HIV-specific T cell responses. The activation of T cells depends on previous antigen processing and engagement by APC (Figure 11). These cells direct the T cell response using different cytokines either in the direction of cell-mediated (Th1) or humoral responses (Th2). The most potent APC are DC, which are able to induce primary and secondary immune responses in both CD4+ and CD8+ T cells (Steinman, Granelli-Piperno et al. 2003, Heath, Belz et al. 2004).

Vacc-4x is a peptide-based therapeutic HIV vaccine candidate consisting of four short HIV p24-like synthetic peptides corresponding to conserved
domains of HIV p24 Gag including HLA-A2 restricting epitopes (Asjo, Stavang et al. 2002, Kran, Sorensen et al. 2004). The peptides are given intradermally together with granulocyte-macrophage colony-stimulating factor (GM-CSF) as a local adjuvant to enhance the maturation of Langerhans cells. In a previous randomised dose-finding phase II clinical study, a cohort of 40 non-AIDS ART-treated HIV-infected patients were given 10 intradermal low or high-dose Vacc-4x injections over 26 weeks followed by two structured treatment interruptions (STI). Vaccine-specific T cell responses were measured \textit{in vivo} by multiple delayed-type hypersensibility skin tests (DTH) and \textit{in vitro} by proliferation and intracellular cytokine staining. Increased Vacc-4x DTH responses were measured in 90% of the immunised patients (Kran, Sorensen et al. 2004, Kran, Sommerfelt et al. 2005, Kran, Sorensen et al. 2006). DTH induration areas were associated with an initial reduction in HIV-RNA setpoint and a slower CD4+ T cell decline, as well as longer time off ART post study (Kran, Sommerfelt et al. 2005). Moreover, Vacc-4x DTH remained stable during STI, in contrast to a reduction in circulating Vacc-4x specific T cells (Kran, Sommerfelt et al. 2005). Finally, no Vacc-4x related escape mutations were observed despite long-term STI (Kran, Jonassen et al. 2010).
6 Aims of the study

The overall objective of this thesis was to study HIV-specific T cell activation and regulation in chronic HIV infection in general, and more specifically in a cohort of patients who were previously immunised with modified HIV p24 Gag consensus sequence peptides (Vacc-4x). The thesis had the following aims:

1. To characterise T cell long-time memory responses in HIV-infected patients on ART from the Vacc-4x vaccination protocol.
2. To evaluate quantitative and qualitative effects of booster-immunisation on T cell activation and regulation in patients on ART from the Vacc-4x vaccination protocol.
3. To study the abundance and clinical relevance of HIV-specific T cell regulation in ART-naïve HIV-infected patients in relation to disease progression.
7 Summary of the papers

7.1.1 Paper I

Intradermal vaccination of HIV-infected patients with short HIV Gag p24-like peptides induces CD4+ and CD8+ T cell responses lasting more than seven years.

Lind A, Sommerfelt M, Holmberg J O, Baksaas I, Sørensen B, Kvale D.

The aim of this paper was to assess T cell memory responses to Vacc-4x. Twenty-two responders from the previous Vacc-4x study, all on effective ART, were retested in median 7.3 years after their last immunisation. Antigen-specific T cell responses to Vacc-4x and native Gag consensus sequence peptides (4xCP) were measured using flow cytometry. A high proportion (95% and 68% respectively) still had Vacc-4x specific CD4+ or CD8+ proliferative T cell responses, which correlated with DTH obtained shortly after completed immunisation more than seven years earlier. Vacc-4x specific INF-γ production from CD4+ T cells and CD107a degranulation from CD8+ T cells were observed in approximately half of the patients. Most patients additionally had CD8+ T cell cross-reacting proliferation to 4xCP that correlated to corresponding Vacc-4x responses.

7.1.2 Paper II

Boosters of a therapeutic HIV-1 vaccine induce divergent T cell responses related to regulatory mechanisms.


The aim of this paper was to assess quantitative and qualitative aspects of T cell responses to reboosts with Vacc-4x. Twenty-five previously immunised patients on effective ART were included. All patients received two
intradermal Vacc-4x booster doses four weeks apart and were followed for 16 weeks. Changes in proliferative responses and IL-10 and TGF-β induced regulation were measured on CD8+ T cells in vitro along with measurements for Vacc-4x specific degranulation (Granzyme B, CD107a) and cytokine production (IFN-γ).

Vacc-4x specific CD8+ T cell proliferation increased in 80% of the patients either after the first (64%) or second (16%) booster. Only 40% still had improved Vacc-4x proliferative responses compared to baseline at the end of the study (week 16). These responders also demonstrated improved CD8+ T cell degranulation, IFN-γ production and DTH. In contrast, the non-responders (60%) had significantly increased IL-10 and TGF-β mediated regulation of Vacc-4x specific T cells. Overall, regulation of Vacc-4x specific CD8+ T cell responses correlated to higher CD8+ T cell PD-1 expression and inversely to CD8+ T cell Vacc-4x proliferation.

7.1.3 Paper III

A parameter for IL-10 and TGF-β mediated regulation of HIV-1 specific T cell activation provides novel information and relates to progression markers.

Lind A, Brekke K, Pettersen F O, Mollnes T E, Trøseid M, Kvale D.

The aim of this paper was to assess cytokine mediated T cell regulation induced by HIV antigens in chronic untreated HIV infection and correlate this to markers of disease progression. Antigen-specific T cell responses to complete HIV Env and Gag 15mer peptides were measured in peripheral blood mononuclear cells (PBMC) from 30 untreated asymptomatic HIV positive patients with and without blocking monoclonal antibodies to IL-10 and TGF-β. The responses of these parallel cultures were used to estimate immune regulation and further related to immune activation (CD38 density
on CD8+ T cells), microbial translocation (LPS levels in plasma) and annual CD4 loss.

T cell regulation \( (R_{AC}) \) was heterogeneous in both specificity and magnitude and not predictable by concurrently measured classic responses. Env \( R_{AC} \) correlated with immune activation and annual CD4 T cell loss rates. Fourteen (47%) of the patients had low \( R_{AC} \) to both Env and Gag. These patients had lower annual CD4 T cell loss and higher CD8 T cell counts than the other patients, and they tended to start ART later than other patients. In contrast, the patients with high Env and Gag \( R_{AC} \) had higher annual CD4 T cell loss rates and lower CD8 T cell counts than other patients.
8 Methodological considerations

8.1 Study design, patient selection, and ethical aspects

All patients were selected from the outpatient clinic at the Department of Infectious Diseases, Oslo University Hospital. Patients for the clinical exploratory trial described in Paper I and II were recruited from a cohort of 38 patients who previously had completed the Vacc-4x immunisation study. The inclusion criteria required the patients to be on effective ART with a viral load <400 copies/ml and to have no clinical signs of immunodeficiency or co-morbidities. Additionally, patients in Paper I had to belong to the Vacc-4x responder group, defined by DTH induration sizes >10 mm² measured at week 38 just after the first STI in the primary immunisation study. This was to ensure that only vaccine responders were included for the assessment of T cell memory responses. All patients in Paper II were informed of possible side effects caused by Vacc-4x immunisation and DTH. They were also informed of the possibility to withdraw from the study without having to specify a reason, and signed informed consents were obtained before the first immunisations were given.

In the study described in Paper III, PBMC from 30 chronically HIV infected treatment naïve patients were selected based on CD38 densities on total CD8+ and CD8+CD38+PD-1+ T cells to cover a wide spectre of HIV associated immune activation.

All studies were approved by the Norwegian South-Eastern Regional Committee for Medical and Health Research Ethics ensuring the studies to be ethical and in accordance with the Helsinki Declaration (World Medical Association 2013).
Factors that may reduce the data’s representativeness for the general HIV-infected population:

- Low number of patients included (21, 25 and 30 patients for Paper I-III respectively) with the risk of making type-2 statistical errors (failure to reject a false null hypothesis, i.e. not being able to demonstrate a true difference between two populations).

- Skewed selection of patients with a high proportion of Caucasian male patients based on the predominance of this patient category in the study cohort. Results do not necessarily apply for women or patients of other ethnicities.

- Paper I and II: selection of only asymptomatic and effectively treated patients, who were responders to Vacc-4x (Paper I). This may represent a bias as only the most immune competent patients with Th1 responses were selected, while less immune competent patients were excluded. Patients with allergic reactions or Th2 directed T cell responses to Vacc-4x were also excluded. Thus, the results from these two studies only account for <53% of the original cohort, and only for the most immune competent patients with primary Th1-type Vacc-4x responses.

### 8.2 Flow cytometry

#### 8.2.1 Background

Flow cytometry has become a powerful tool in medical science, providing the opportunity to study different immunological aspects on a single cell level (Perfetto, Chattopadhyay et al. 2004). This application has also made it possible to gain insights into key immune pathogenic mechanisms in HIV (Chattopadhyay and Roederer 2010). The most advanced modern multi-laser flow cytometers can quantify up to 10,000 cells with 18 different cell properties per second, due to remarkable improvements in hardware and
software technology and a steadily growing number of available fluorochromes and monoclonal antibodies.

8.2.2 Basic principles

Through hydrodynamic focusing, the cells are brought into a single line within a laminar flow of saline solution (sheath fluid), before they intercept a laser beam. The cells scatter the laser light depending on structure and size, which is recorded by photo multiplying tubes (PMT). The amount of light scattered at small angles is proportionate to the cell size and is recorded by the forward scatter PMT (FSC) placed at a straight angle. Light scattered at larger angles depend on the cell’s inner complexity and is recorded by the perpendicularly placed side scatter PMT (SSC). By FSC and SSC alone, it is possible to discriminate between for example erythrocytes, lymphocytes, granulocytes and monocytes (Figure 12).

To differentiate further between cell types or to study cell receptors and functions, cells are labelled with fluorochrome-conjugated monoclonal antibodies (mAbs) (Bogh and Duling 1993, McLaughlin, Baumgarth et al. 2008). Fluorochromes emit light at characteristic colours and intensities (emissions peaks) when excited by a laser. The emitted light is recorded by dedicated PMTs (also called channels). PMT-data are processed by a computer and presented graphically as histograms or dot plots and further analysed using Boolean gating. Thus, by combining different specific fluorochrome-conjugated antibodies and intracellular fluorescent dyes in multicolour assays, it is possible to study cell subsets on a receptor level, for example to measure CD4+ and CD8+ T cell proliferation and activation, to enumerate INF-γ or CD107a producing cells or to quantify CD38 densities on CD8+ T cells.
8.2.3 Pitfalls

When conducting flow cytometry, it is important to be aware of several pitfalls, which can otherwise lead to erroneous results. First, as fluorochromes emit light over a certain spectrum, spectra from different fluorochromes can overlap even when optimised band pass filters are used. The amount of overlap or “spill-over” from one fluorochrome to another fluorochrome’s PMT is revealed by single-stained cells and automatically compensated for in modern flow cytometer software. Still, one must recognise and consider possible spillover effects, as even software-calculated compensations can be incorrect. Second, the amount of fluorescence or scattered light recorded by the different PMTs can fluctuate over time due to variations in laser power, PMT sensibility and fluorochrome quality. This “drifting” can profoundly influence results in longitudinal studies and must be accounted for. In the BD FACS Canto II cytometer used in this study, longitudinal drifting is recorded and automatically compensated for with daily runs of so-called Cytometer Setup and Tracking beads. A third important factor that can critically affect the results in flow
cytometry is the decision of where to set the cut-off between positive and negative events. This is even more difficult when using markers with continuous ranges of expression such as CD25, PD-1 and HLA-DR or when analysing dull-stained subsets. In such cases fluorescence-minus-one (FMO) is helpful and was used throughout this study (Maecker and Trotter 2006). Here, negative control cells are stained with all fluorescent colours except the colour in question and thereby objectively defining where to expect the maximum fluorescence spillover for that given channel (Baumgarth and Roederer 2000).

8.3 ELISPOT

Results from numerous HIV vaccine studies are partly or exclusively based on IFN-γ measurements by Enzyme-linked ImmunoSpot Assay (ELISPOT), which is a sensitive, highly quantitative, and inexpensive method to assess specific antigen responses (Reviewed in Slota, Lim et al. 2011). Cytokine-specific monoclonal antibodies are coated onto nitrocellulose or polyvinylidene fluoride membranes. Binding of the locally produced cytokine is visualised by detection-antibodies conjugated to spot-forming substrates. Each spot represents one cytokine-producing cell and is counted manually or by ELISPOT readers connected to analysing software.

ELISPOT has several limitations that have to be considered when interpreting data. The number of spots produced depends on the number of viable cytokine producing cells present and can vary between culture wells. The threshold used for size and intensity when defining spots is subjective and can influence results. Variations between ELISPOT plates can occur due to inaccuracies in production and in how the assay is performed. Still, the most important limitation of ELISPOT is the inability of the assay to discriminate between different cell subsets that produce the same cytokine. This can partly be achieved by pre-assay cell depletion or enrichment or resorting to FlouroSpot, but the subset analysing possibilities remain inferior to that of flow cytometry.
In Paper II, ELISPOT was used to quantify Granzyme B degranulating cells in antigen-stimulated PBMC to evaluate changes in vaccine-induced CD8+ T cell killing capacity. To rule out inter-assay variation, the assay was done in one run. Viable cells were counted by flow cytometry to ensure equal numbers of CD8+ T cells in all wells for each patient and timepoint. An ELISPOT reader was used to count median spot values from duplicate control and triplicate antigen-stimulated wells.

### 8.4 ELISA, LAL and Luminex Multiplex assays

Enzyme-linked immunosorbent assay (ELISA) is frequently applied for the detection and quantification of a wide array of biological agents like proteins, cytokines, viruses and antibodies. It is inexpensive, highly specific, sensitive, flexible and easy to conduct (Gan and Patel 2013). Specific antibodies coated on the bottom of microtitre plates bind the analyte of interest. Antibody-analyte complexes are then linked directly or indirectly to an enzyme that catalyses a reaction in a detection substrate leading to colour changes. The degree of colour change is proportionate to the amount of analyte present and is measured by a photometer.

The Limulus amebocyte lysate (LAL) assay is also a highly sensitive and widely used assay based on substrate dependent colour changes (Murer, Levin et al. 1975). LAL, which is extracted from white blood cells from the American horseshoe crab (*Limulus polyphemus*), cleaves a chromophore in the presence of LPS. This causes a yellow colour to develop, which is proportionate to the LPS present.

The Luminex multiplex technology combines ELISA and flow cytometry and uses different sized and fluorescent-coloured beads, which are specific to different cytokines (Vignali 2000). This makes it possible to precisely and rapidly to perform up to 100 unique cytokine measurements within a single small-volume sample (Elshal and McCoy 2006).
In Paper II and III, ELISA and Luminex were used to quantify cytokines and chemokines in snap-frozen plasma and in supernatants from *in vitro* proliferation assays, whereas the LAL-assay was performed to quantify LPS in plasma.

An important limitation for all these methods is that the estimated absolute concentrations cannot readily be compared within or between the different assays. Samples are diluted depending on the initial concentration of the analyte and the working range of the assay. Immune precipitation and reactions to agents like EDTA are factors that reduce the precision.

### 8.5 T cell Assays

#### 8.5.1 Fresh drawn cells versus the use of cryopreserved cells

In Paper I only fresh PBMC were used whereas fresh and/or cryopreserved PBMC were used in Papers II and III. After thawing and washing, the cells were reconstituted overnight before their viability was tested using microscope and Tryptan Blue staining. Only PBMC with viability above 85% were accepted for further culturing. The use of cryopreserved cells has many advantages but also important disadvantages (Table 2). Some cell subsets or cell functions may succumb to cryopreservation, a possibility that is even more likely in untreated HIV where most cells are highly activated and theoretically more fragile (Reimann, Chernoff et al. 2000).
Table 2.

<table>
<thead>
<tr>
<th>Pro and contra of cryopreservation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pro</strong></td>
</tr>
<tr>
<td>Reduces operator- and hardware dependent inter-assay variability and day-to-day variation.</td>
</tr>
<tr>
<td>Optimises the use of available resources.</td>
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<tr>
<td>Opens up for multicentre studies with centralised laboratory facilities.</td>
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<tr>
<td>Less labour intensive and easier logistics through batch analyses.</td>
</tr>
<tr>
<td><strong>Contra</strong></td>
</tr>
<tr>
<td>Higher cell loss through additional cell handling and centrifugation steps.</td>
</tr>
<tr>
<td>Cryopreservation-induced cell death may affect results.</td>
</tr>
<tr>
<td>Reduced expression of receptors such as PD-1, CD38, and HLA-DR (Holm, Pettersen et al. 2008).</td>
</tr>
<tr>
<td>Changed/decreased cell reactivity of some cell subsets?</td>
</tr>
</tbody>
</table>

8.5.2 Markers of T cell functions

Specific T cell responses are diverse both in quality and quantity. Typically, three response qualities are estimated in vitro: activation, proliferation and cytotoxicity (degranulation). Furthermore, soluble cytokines are frequently measured as these indirectly reflect response patterns such as Th1 and Th2. By measuring multiple response parameters to the same antigen at a single-cell level, polyfunctionality can be estimated.

Assessing altered T cell responses to HIV antigens may elucidate immune pathogenic processes also important for the development of immune therapies. Estimating immune activation and T cell senescence however, may help categorise patients into clinical relevant progressor groups (Douek, Roederer et al. 2009).
8.5.2.1 Activation

T cell activation is the first step of the antigen-specific response, followed by clonal expansion (proliferation), differentiation and production of cytokines. Various surface markers are only expressed or significantly upregulated on T cells during activation (Caruso, Licenziati et al. 1997). The earliest activation marker is CD69 followed by the transferrin receptor CD71 and the IL-2 receptor CD25. Ligation between CD25 and IL-2 stimulates growth, differentiation and survival of antigen-specific CD8+ T cells. In long-term cultures, enhanced expression of CD25 defines a newly proliferated cell population and not all proliferated cells (Fazekas de St Groth, Smith et al. 2004). Later on, HLA-DR is upregulated. This is a MHC class II molecule only expressed on T cells during activation (Reddy, Eirikis et al. 2004).

Another activation marker is CD38 (cyclic ADP ribose hydrolase), which is expressed on various cell subsets including naïve and activated T cells. During increased T cell proliferation and turnover, the expression of CD38 is relatively increased in peripheral blood. In chronic HIV, CD38 expression reflects immune activation and serves as a marker for disease progression (Holm, Pettersen et al. 2008, Chattopadhyay and Roederer 2010).

8.5.2.2 Proliferation

An important property of effective T cell clones is their ability to proliferate. This has traditionally been measured using the radioactive nucleoside $^3$H-thymidine (Corradin, Etlinger et al. 1977). Proliferating cells incorporate $^3$H-thymidine in their chromosomal DNA. The extent of cell division is estimated by the amount of radioactivity in the DNA recovered from the cells. Major drawbacks are radioactivity, and that the assay does not provide any phenotypic information. A similar method is the detection of incorporated 5-bromo-2′-deoxyuridine (BrdU), but this method also allows for the phenotypic characterisation of dividing cells (Dolbeare, Gratzner et al. 1983, Rothaeusler and Baumgarth 2007). This is also true for the nuclear protein Ki-67. Ki-67 is
expressed during all phases of cell division and DNA repair, but not in quiescent cells, and can therefore be used to define both proliferation and cell turnover (Soares, Govender et al. 2010).

Labelling cells with the fluorescent cytosol dye carboxyfluorescein succinimidyl ester (CFSE) is currently the most frequently applied method for measuring proliferation. The precursor CFDA-SE diffuses into the cell and is transformed into CFSE by esterases in the cytosol. CFSE binds covalently to intracellular molecules and remains intracellular even during cell division. As only half of the CFSE labelled cytosol is passed onto each daughter cell, the fluorescence intensity is reduced by 50% after each cell division (Figure 13). By using CFSE in multicolour flow cytometry, it is possible to identify up to eight discrete cell generations on a subset level and study other cell functions within these generations (Lyons 2000).

A potential problem with CFSE is its known time and concentration dependent cytotoxicity during staining (Chattopadhyay and Roederer 2010). Although generally supposed to be negligible, cytotoxicity may still have an impact on results, since various cell subsets could be affected differently. This should especially be considered when CFSE is used on fragile cells like cryopreserved PBMC from fast progressing untreated HIV patients. Also, since CFSE fluorescence is very bright with a very broad spectre, a more careful optimisation of the flow cytometer is needed to avoid spillover effects.
Figure 13. Schematic illustration of the successive halving of intracellular CFSE after each cell division (the left panel) also leading to equivalent halving of fluorescence intensity measured by flow cytometry (the right panel) (Figure adapted from Lyons and Parish 1994).

8.5.2.3 Cytotoxicity

Cytotoxicity represents the main function of CD8+ T effector cells, namely the killing of infected target cells. This is mediated through the degranulation of perforins and granzymes into the immunological synapse, which is the space between the T cell and its target (Betts, Brenchley et al. 2003).

A classical way to assess cytotoxicity is the $^{51}$Chromium ($^{51}$CR) release assay (Shacklett 2002). Principally, target cells are labelled with radioactive $^{51}$Cr before incubation with effector cells. If target cells are lysed by antigen-specific effector cells, $^{51}$Cr is released into the culture medium. The amount of $^{51}$Cr measured in the medium therefore reflects the level of antigen-specific killing. The main drawbacks of this assay are the involvement of a radioactive reagent, a low sensibility due to spontaneous $^{51}$Cr release, and its inability to provide any phenotypic information.

A more convenient marker for cytotoxicity is the lysosomal associated membrane glycoprotein-1 (CD107a), which is integrated in the lipid layer surrounding the cytotoxic granule. During degranulation, CD107a is temporarily expressed on the cell surface. By adding fluorochrome-labelled CD107a before stimulation, it is possible to assess antigen-specific
degranulation by CTL (i.e. killing capacity) as well as additional phenotypic information (Betts, Ambrozak et al. 2001).

Finally, the protease Granzyme B is released by CTL upon target cell binding leading to apoptosis. Granzyme B release can be assessed directly by ELISPOT, and is a sensitive indicator of CTL activity (Shafer-Weaver, Sayers et al. 2003).
8.5.2.4 *T cell response parameters used in Papers I-III*

Various response parameters were chosen in the papers included in this thesis to assess a broad scope of T cell responses. Proliferation was the main readout (see Table 3).

**Table 3. T cell markers applied in paper I-III**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Characteristics</th>
<th>Paper</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFSE</td>
<td>Proliferation.</td>
<td>I, II</td>
<td>See chapter 8.5.2.2.</td>
</tr>
<tr>
<td>CD25</td>
<td>IL-2 receptor, upregulated upon activation.</td>
<td>III</td>
<td>Also upregulated on unspecific activated T cells and Treg.</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>Upregulated on T cells upon activation.</td>
<td>I-III</td>
<td>Also upregulated on unspecific activated cells.</td>
</tr>
<tr>
<td>CD107a</td>
<td>Degranulation marker (see chapter 8.5.2.3).</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>Granzyme B</td>
<td>Degranulation marker (see chapter 8.5.2.3).</td>
<td>II</td>
<td>Also produced by NK cells.</td>
</tr>
<tr>
<td>INF-γ production</td>
<td>Promotes Th1 differentiation and development of cytotoxic T cells.</td>
<td>I, II</td>
<td>Also produced by other cell subsets like myeloid cells, DC and macrophages.</td>
</tr>
<tr>
<td>Cytokines in supernatants or plasma</td>
<td>Cytokine production of T cells and other cell subsets.</td>
<td>II, III</td>
<td>Not cell-specific. Indirect measure of T cell responses.</td>
</tr>
<tr>
<td>PD-1</td>
<td>Immune senescence, T cell regulation.</td>
<td>I-III</td>
<td></td>
</tr>
<tr>
<td>CD38 density on CD8+ T cells</td>
<td>Immune activation.</td>
<td>III</td>
<td></td>
</tr>
<tr>
<td>DTH</td>
<td>Antigen-specific CD4+ T cell responses <em>in vivo</em>.</td>
<td>I, II</td>
<td></td>
</tr>
</tbody>
</table>
8.5.3 Cell culturing time

Proliferation was performed over six days based on pre-trial testing of 4, 6, 8, 10 and 14 days assays. In general, long-time assays have the following benefits and disadvantages (Table 4):

Table 4.

<table>
<thead>
<tr>
<th>Pro and contra of long-time cell cultures</th>
</tr>
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<tbody>
<tr>
<td><strong>Pro</strong></td>
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<tr>
<td></td>
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<tr>
<td><strong>Contra</strong></td>
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</tbody>
</table>
8.5.4 Definition of response

Responses were defined as the expression of the marker or markers in question above those obtained from unstimulated control wells with otherwise similar conditions (Figure 14). **Responders** in Paper II were defined as patients with increased CD8+ T cell responses to Vacc-4x compared to their respective baseline responses. Gating strategy can affect the response read-out in flow cytometry (see chapter 8.2.3) and should always be included when presenting flow cytometry derived data (Lee, Spidlen et al. 2008).

![Figure 14. Methods of measuring antigen-specific T cell proliferation and activation on viable cells by flow cytometry](image)

Figure 14. Methods of measuring antigen-specific T cell proliferation and activation on viable cells by flow cytometry (orange transparent boxes). The left plot shows proliferation of T cells during antigen stimulation defined by CFSE median fluorescence intensities equal to or below the second proliferated generation. The centre plot shows activated T cells concurrently expressing the two activation markers HLA-DR (x-axis) and CD25 (y-axis). In paper III, the coexpression of HLA-DR and CD25 above background was assessed to estimate antigen-specific T cell responses. The right plot shows both proliferating (i.e. CFSE dim, x-axis) and activated (i.e. HLA-DR positive, y-axis) T cells as applied in papers I and II (frequency (%) indicated). In long-time cultures, proliferation and activation parameters reflect overlapping aspects of antigen-specific T cell responses, since antigen-specific activation is closely followed by clonal expansion (Reddy, Eirikis et al. 2004, Lind, Brekke et al. 2014, Figure 1). (Figure by Andreas Lind, 2013).

8.6 Quantifying immune regulation

In classical T cell assays such as proliferation or activation, the impact of immune regulation remains largely unknown and is rarely addressed, since normally only the final response is measured. Subject A and B can both have a similar response to a given antigen, but still have very different levels of immune regulation.

To quantify the effects of immune regulation on an individual basis, we applied blocking mAbs against IL-10 and TGF-β. These regulatory cytokines where targeted due to their central role in T cell regulation (see chapter 5.7.3). The
differences in antigen-specific responses between parallel wells with or without blocking mAbs to IL-10 and TGF-β were used to estimate the effect that these cytokines have on the final antigen-specific T cell response (Figure 15).

Figure 15. Quantitative measure for IL-10 and TGF-β induced T cell regulation. A. Schematic outline of the assay quantifying antigen-specific IL-10 and TGF-β induced T cell regulation. The left panel shows a conventional T cell assay and the right panel shows a possible outcome when blocking T cell regulatory cytokines. T cell regulation (Δ) was defined as the difference in responses to the same antigen between these panels. B. Example of two study patients from Paper II with similar conventional CD8+ T cell responses (the left upper and middle panels) to Vacc-4x peptides (0.95% and 0.99%, respectively) above corresponding unstimulated (negative) controls. Parallel cultures with anti-IL-10 and anti-TGF-β blocking mAbs reveal moderately increased (Δ = +0.53%, subject #10, the right upper panel) or strongly increased (Δ = +3.04%, subject #23, the right middle panel) responses after correcting for respective negative controls. The two lower panels show negative controls for subject #23 in the absence (the left lower panel) or presence (the right lower panel) of anti-IL-10 and anti-TGF-β blocking mAbs, respectively. Note a typical increase in background proliferation (CFSE diminution, x-axis) and activation (HLA-DR+, y-axis) during IL-10 and TGF-β blockade (Figure adapted from Lind, Brekke et al. 2013).

8.7 Statistics

Non-parametric tests are chosen when the variables in question are not normally distributed as often is the case for biological data or data derived from small sample sizes (Panda, Chen et al. 2013). Normal distribution can sometimes be achieved by log transformation. If this fails non-parametric tests must be applied. In the papers included in this thesis, Mann-Whitney U and Kruskal-Wallis tests were performed to analyse differences between two or
more independent groups. The Wilcoxon signed-rank test was used to analyse
dependent variables, the Spearman Rank for correlation analysis, and the Fisher
exact test for analysing cross-tabulated data.
9 Discussion

9.1 An individualised immunisation approach

Therapeutic HIV vaccines have so far failed to show lasting and positive effects on “hard” clinical endpoints as discussed earlier. The reason for this is probably multifactorial and interlaced. The nature of vaccine-induced responses depends on various known and unknown factors, including vaccine-dependent and individual aspects. First, a full and comprehensive understanding of the nature of the immune response responsible for protection is still missing (Virgin and Walker 2010, Garcia, Leon et al. 2012). Second, there is a lack of good immunologic markers that reflect vaccine efficacy in vivo (Garcia, Leon et al. 2012). Third, the optimal combination of antigen, immunisation method (peptides, DNA, pulsed DC), location (intradermal, mucosal), frequency (prime-boost, multiple immunisations, late-boost), adjuvant and/or vector is unknown. A fourth factor may be that the therapeutic HIV vaccine must overcome the immune suppressive environment caused by chronic immune activation and not induce inhibition itself, a factor that may be connected with the above-mentioned points (Brooks, Lee et al. 2008, Ha, Mueller et al. 2008, Pettersen, Tasken et al. 2010, Porichis and Kaufmann 2012).

Due to individual differences in responses to HIV and immunisation, it is most likely that different patients will profit more or less from vaccination, and that some patients will be better off without a vaccine. Just as important as finding the right vaccine is to define who should receive a given vaccine and how. Until these questions are solved, vaccine trials will remain empirical or have to rely on immunological pseudomarkers with uncertain clinical relevance.

After the first Vacc-4x study, it became apparent that responses to immunisation varied greatly both in character and magnitude and seemed to depend on antigen dose and HLA haplotype (Kran, Sorensen et al. 2004). In the papers included in this thesis, we tried to elucidate some aspects of
immunisation: a) the generation of long-lasting memory responses in relation to dose and HLA, b) the boosting of such responses several years later, and c) the impact of T cell regulation during reboost. Our current findings support the notion that immunisation regimens must be individualised to achieve the required result.

**Vaccine dose and HLA haplotype:** It has been shown that low-peptide stimulation generates more sensitive T cells than high-dose stimulation (Alexander-Miller, Leggatt et al. 1996). Low-dose vaccination has shown to induce polyfunctional T cells more efficiently in mice (Darrah, Patel et al. 2007). In paper I, we demonstrated that a majority of patients still had proliferative and cytotoxic responses to Vacc-4x and corresponding “natural” p24 Gag peptides more than seven years after primary immunisation (Lind, Sommerfelt et al. 2012). These responses seemed to depend both on vaccine dose and HLA-A2: patients who had received low dose vaccination and who were HLA-A2 positive had better T memory responses and cross-reactivity than other patients. This may be explained by a superior induction of Th1 directed memory responses in these patients.

**Frequency:** Repetitive immunisations may induce immune regulation instead of reactive memory responses (Zhou, Drake et al. 2006, Petalas and Durham 2013, Honda, Egen et al. 2014). In paper II, where the study cohort consisted of patients who previously had received repetitive immunisations including multiple DTHs, responses to reboost were highly variable. Eighty per cent responded either after the first (64%) or the second (16%) booster dose. Vaccine related T cell regulation could explain the failure in boosting the majority of patients (60%); T cell regulation by IL-10 and TGF-β increased in non-responding patients during immunisations and seemed to hamper re-immunisation outcome.

The **route of immunisation** may influence both the effectiveness and focus of responses. It has been shown that mucosal vaccines induce T cells with higher
antigen sensibility than subcutaneous immunisation, probably due to the presence of a higher number and more effective APC in mucosal compartments than in the skin (Belyakov and Ahlers 2012). This aspect is currently studied in relation to Vacc-4x in an ongoing clinical trial by Brekke et. al (paper currently in preparation).

When interpreting the results from paper I and II, one has to be aware of several limitations. First, both studies were performed on a small and inhomogeneous group of individuals who previously had received Vacc-4x vaccination. Second, the primary immunisation regimen had consisted of multiple intradermal injections and DTHs with possible induction of tolerability not necessarily applicable for other immunised cohorts. Other limitations are patient selection and the methodical considerations mentioned in chapter 8.1. Strictly speaking, these findings only apply for the peptide-based vaccine candidate Vacc-4x and only in relation to antigen-specific CD8+ T cell in vitro responses. In relation to vaccine efficacy, it remains unclear whether responses to Vacc-4x or cross-reactivity to natural peptides are of any relevance in relation to effective HIV immunity. Reboost did not affect clinical end-points such as CD4 and CD8 counts or disease progression. This was as expected in an ART treated cohort within a short study period. Still, ultrasensitive measurements of HIV RNA during reboost could have revealed or dismissed a possible clinical effect. Furthermore, it could be claimed that current HIV p24 responses measured in Paper I had been induced by viraemia during STI, since the study did not include a placebo arm. A strong argument against this notion is that 91% of the patients showed no signs of peptide-specific DTH responses before primary immunisation, and that these responses developed only after Vacc-4x immunisation had started (Lind, Sommerfelt et al. 2012, Figure 2).

Despite these limitations, it seems clear that not all patients should have received the same immunisation regimen. This is especially apparent for the patients who had a consecutive reduction of their Vacc-4x responses after the second reboost. For Vacc-4x, this calls for individualised immunisation
regimens using differentiated doses, frequencies and HLA-matched vaccine recipients. This includes methods to identify patients who are going to profit from immunisation before inclusion. Patients who profited the most from reboost in paper II had higher CD8+ T cell degranulation and CD4+ INF-γ production at baseline than non-responders, whereas non-responders had higher production of proinflammatory and regulatory cytokines than responders (Lind, Brekke et al. 2013). Whether these parameters could have been used to pinpoint responders before reboost can be rightfully questioned and calls for further investigation.

Diverse responses to immunisation will likely apply for other therapeutic HIV vaccines as well. Other vaccines will also induce variable responses related to dose, frequency, haplotype, and immune regulation.

The importance of immune regulation for vaccine efficacy has also been described elsewhere (Ha, Mueller et al. 2008, Macatangay, Szajnik et al. 2010, Porichis and Kaufmann 2012). However, our or similar exploratory parameters for T cell regulation have to our knowledge not been tested in conjunction with a therapeutic HIV vaccine. We demonstrated that T cell regulation varied at baseline and was induced differently between patients. Ultimately, T cell regulation seemed to hamper the effects of immunisation. This strongly suggests that immune regulation must be accounted for in future vaccine studies.

9.2 Immune regulation in chronic untreated HIV infection

Potentially effective HIV-specific T cell responses are not only critically weakened by the early loss of HIV-specific CD4+ T cells, viral immune escape and immune activation, but probably also directly hampered by immune regulation, as discussed in chapter 8.5.2. Studying the mechanisms, coordination and development of these regulating factors will be essential for a better understanding of HIV pathogenesis and for the development of effective immune therapies. However, it is equally essential to understand how these
immune regulating factors influence the quality and strength of HIV-specific T cell responses. Thus, to quantify immune regulation as an “effector function” will provide important information, even when the underlying mechanisms are unknown. This information is not revealed by classical T cell activation assays, since these assays only reflect net activation with regulation as an unknown contributing factor.

In paper III we attempted to quantify the final effect of T cell regulation and relate this to disease progression in chronic untreated HIV infection. We chose the same assay as in paper II, and quantified the regulation of Env and Gag specific T cell responses. We demonstrated that IL-10 and TGF-β induced T cell regulation was HIV antigen-specific and highly variable between patients. The magnitude of regulation was very high in some individuals and was not predicted by the corresponding, classical activation parameter. High immune regulation seemed clinically unfavourable. Patients with a high regulation of Env and Gag responses had a more rapid disease progression in terms of annual CD4 T cell loss and immune activation. Thus, quantifying the final effects of immune regulation seemed to reflect processes that were related to disease progression.

Our approach of assessing T cell regulation has several theoretical disadvantages. Obvious limitations are assay variability, response definitions, and a small study cohort. The assay was performed on PBMC in vitro and the results do not necessarily apply in vivo or for different compartments such as lymphatic tissue and mucosa. It remains unknown how IL-10 and TGF-β induced immune regulation affects other T cell response modalities such as cytotoxicity or polyfunctionality. How IL-10 or TGF-β alone affects T cell responses is not exposed, nor is how this may vary between patients. It also remains unknown which up-stream immunological players are involved and the sources of these cytokines. Finally, it can be questioned whether our definition of regulation is relevant, since only the effects of IL-10 and TGF-β were assessed. The assay does not reveal other immune regulatory mechanisms such
as Treg, CTLA-4 or PD-1, mechanisms that perhaps even are compensatory changed during IL-10 and TGF-β blockade.

However, the potential of our assay is that it assesses a central pathway of immune regulation. The assay is relatively simple to conduct and therefore applicable for larger study cohorts. Information from such an assay may spark new ideas for further research on immune regulation in HIV and other chronic viral infections. This may contribute to a better understanding of the complex interplay between regulation and activation, and help determine the significance of regulation on prognosis and in relation to immune therapy.
10 Conclusions

1. Vacc-4x was able to induce long-time T cell memory clones with proliferative and cytotoxic capacity in HIV-infected ART patients.

2. Vacc-4x booster-immunisation induced:
   - improved proliferative T cell responses with cytotoxic capacity and INF-γ production in some patients.
   - IL-10 and TGF-β related regulation of T cell responses that seemed to hamper the booster effect in a majority of patients.

3. In chronic untreated HIV infection, IL-10 and TGF-β induced regulation of HIV-specific T cell responses:
   - were HIV-antigen specific, and unevenly distributed in magnitude.
   - were not revealed by the conventional T cell assay.
   - seemed to define patients with faster disease progression.

Future trials must explore the relevance of immune regulation for therapeutic vaccine efficacy and for the assessment of HIV infected patients.
The year 2013 marked the 30th anniversary of the publication by Barré-Sinoussi et al describing the first isolation of HIV-1 (Barre-Sinoussi 1983). Since then, intense research has resulted in important discoveries that have helped to understand the nature of the HIV pathogenesis (Barre-Sinoussi, Ross et al. 2013). These discoveries have also led to the development of new antiretroviral drugs. Despite this, many key aspects of HIV remain elusive, and we have no cure. ART effectively reduces disease progression, but it seems unlikely that ART alone can eradicate HIV, or that it will be possible to provide life-long ART coverage for all infected patients (Butler, Valdez et al. 2011, Lewin and Rouzioux 2011, Hamimi, Pancino et al. 2013).

Effective immune therapies would clearly be a better alternative to life-long ART. It is therefore crucial to further identify the mechanisms for immune protection in HIV and how these can be induced (Porichis and Kaufmann 2012). Therapeutic vaccine studies may in this setting give important clues (Burton, Ahmed et al. 2012). Currently, it seems that effective vaccines will need to induce coordinated responses from B and T cells as well as innate immune responses (McElrath and Haynes 2010, Alter, Heckerman et al. 2011). Still, the biggest challenges remain virus diversity and escape from immune responses, latent viral reservoirs, and the lack of good immune correlates for vaccine efficacy (Amanna, Messaoudi et al. 2008, Garcia, Leon et al. 2012, Borthwick, Ahmed et al. 2013).

Therapeutic vaccine studies may also be a more ethical way to identify antigen candidates for prophylactic HIV vaccines. Therapeutic vaccine trials are conducted on much smaller cohorts and are therefore cheaper, less complicated and more effective than prophylactic vaccine trials. The RV144 prophylactic vaccine was tested on more than 16000 HIV negative patients who were not offered any other protection than counselling (Rerks-Ngarm, Pitisuttithum et al. 2009). Other prophylactic HIV vaccine studies, including the recently
terminated DNA/rAd5 study, were also conducted on big cohorts and relied on healthy volunteers at risk of HIV infection (Hammer, Sobieszczyn et al. 2013). This raises the question whether it is unethical to perform costly large-scale vaccine testing without first offering standard of care treatment.

In the future, HIV treatment will probably not only consist of combination antiretroviral therapy, but also consist of a “combination immune therapeutic treatment”. Since the host’s immune system per definition is insufficient to control viral replication, it is probable that some form of therapeutic vaccination will be included within a regimen for cure. An example is intensified ART combined with reactivation strategies to target latent viral reservoirs (Lehrman, Hogue et al. 2005, Vanham and Van Gulck 2012).

To speed and improve vaccine development, individualised approaches and broad immune surveillance before and during immunisation will be necessary. Vaccine efficacy may also be improved by additionally modulating immune regulation and exhaustion. Examples of this are PD-1 or IL-10 blockade (Ha, Mueller et al. 2008, Porichis and Kaufmann 2012, Porichis, Hart et al. 2013), or immune modulatory agents such as lenalidomid (Bodera and Stankiewicz 2011, Henry, Labarte et al. 2013) (ongoing trial with Vacc-4x) and Cox-2 inhibitors (Pettersen, Torheim et al. 2011).
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with early plasma viral load, CD4+ cell count and rate of progression to AIDS following acute HIV-1 infection. Multicenter AIDS Cohort Study." Aids 12(16): 2107-2113.


13 Papers I-III
Boosters of a therapeutic HIV-1 vaccine induce divergent T cell responses related to regulatory mechanisms

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A B S T R A C T

Therapeutic human immunodeficiency virus (HIV) vaccines aim to reduce disease progression by inducing HIV-specific T cells. Vacc-4x are peptides derived from conserved domains within HIV-1 p24 Gag. Previously, Vacc-4x induced T cell responses in 90% of patients which were associated with reduced viral loads. Here we evaluate the effects of Vacc-4x boosters on T cell immunity and immune regulation seven years after primary immunization. Twenty-five patients on effective antiretroviral therapy received two Vacc-4x doses four weeks apart and were followed for 16 weeks. Vacc-4x T cell responses were measured by proliferation (CFSE), INF-γ, CD107a, Granzyme B, Delayed-Type Hypersensitivity test (DTH) and cytokines and chemokines (Luminex). Functional regulation of Vacc-4x-specific T cell proliferation was estimated in vitro using anti-IL-10 and anti-TGF-β monoclonal antibodies.

Vacc-4x-specific CD8+ T cell proliferation increased in 80% after either the first (64%) or second (16%) booster. Only 40% remained responders after two boosters with permanently increased Vacc-4x-specific proliferative responses (p = 0.005) and improved CD8+ T cell degranulation, IFN-γ production and DTH. At baseline, responders had higher CD8+ T cell degranulation (p = 0.05) and CD4+ INF-γ production (p = 0.01), whereas non-responders had higher production of proinflammatory TNF-α, IL-1α and IL-1β (p < 0.045) and regulatory IL-10 (p = 0.07).

Notably, IL-10 and TGF-β mediated downregulation of Vacc-4x-specific CD8+ T cell proliferation increased only in non-responders (p < 0.001). Downregulation during the study correlated to higher PD-1 expression on Vacc-4x-specific CD8+ T cells (r = 0.44, p = 0.037), but was inversely correlated to changes in Vacc-4x-specific CD8+ T cell proliferation (r = −0.52, p = 0.012).

These findings show that Vacc-4x boosters can improve T cell responses in selected patients, but also induce vaccin-specific downregulation of T cell responses in others. Broad surveillance of T cell functions during immunization may help to individualize boosting, where assessment of vaccine-related immune regulation should be further explored as a potential new parameter.

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1. Introduction

Human immunodeficiency virus (HIV) type 1 preferentially targets and depletes two types of activated CD4+ T cells in primary infection; CCX5/CD4+ Th17 mucosal T cells [1] and subsequently HIV-specific CD4+ T cells. The latter clones are essential in maintaining effective viral control in collaboration with CD8+ T cells of the same specificities [2,3]. In chronic HIV infection there is an altered balance between immune activation, immune exhaustion and regulation. Recent data suggests that low T regulatory cell (Treg) responses are associated with viral control in HIV controllers [4] and that protective HIV-specific CD8+ T cells evade Treg suppression [5].

Therapeutic HIV vaccines aim to repair HIV-specific cellular immunity after primary infection by inducing new T cell clones and thereby better control viral replication, especially through polyfunctional HIV-specific CD8+ T cell responses [6,7]. Thus, effective vaccination may potentially reduce disease progression, delay the initiation of antiretroviral treatment (ART) and help to decrease residual viral load levels in already treated patients [8].

Vacc-4x is a therapeutic HIV-1 vaccine candidate derived from conserved domains within p24 Gag, designed to stimulate cellular immune responses [9]. Previously, Vacc-4x stimulated proliferative T cell responses in 90% of immunized HIV positive patients [10]. Enhanced T cell responses to Vacc-4x were associated with reduced viral loads during ART interruptions [11,12] and postponed reinduction of ART [10] without inducing viral immune escape [13]. Furthermore, we have demonstrated long-term Vacc-4x-specific T cell memory in the same cohort [10,14].

The objective of this study was to evaluate how two booster immunizations of Vacc-4x would influence quantitative and qualitative aspects of Vacc-4x-specific T cell immune responses in previously immunized subjects, with the ultimate goal to explore the potential of developing individualized immunization regimens for HIV-infected patients. Although vaccine boosters aim to strengthen the pool of effector T cells, boosting might theoretically also induce or enhance a spectrum of immune regulatory mechanisms [15,16]. However, to our knowledge, regulation has not been assessed during therapeutic HIV vaccine boosters in man. We hypothesized that responses to two consecutive boosters would be differentiated and that immune regulatory mechanisms might play a role. We assessed the regulation of vaccine-specific T cell proliferation by the two key inhibitory soluble cytokines IL-10 and TGF-β in vitro [16,17]. Instead of characterizing the many possible cellular sources for these cytokines, we here examined their collective influence on T cell effector function in the individual patient. We found that changes in this parameter could explain the diversified responses to booster vaccination.

2. Materials and methods

2.1. Study participants and study design

Twenty-five HIV positive patients who had completed the first Vacc-4x study and who had been on effective ART for at least 6 months, with viral load <20 copies/ml and with no clinical signs of immune deficiency were included for re-immunization in this non-randomized single-center, open-label one arm study. Patients with previous allergic reactions to Vacc-4x, malignant disease, immune suppressive therapy, concurrent active infections, as well as pregnant or breastfeeding women were excluded. Fifteen minutes prior to immunizations, low dose GM-CSF (Leukine; Genzyme, MA, USA) was injected intradermally followed by 100 µl Vacc-4x (300 µg/ml per peptide) intradermally in the same spot at baseline and after 4 weeks. Clinical examination, general biochemistry and hematologic analyses, HIV-RNA, CD4 and CD8 cell counts were performed on every visit at study weeks 0, 4 and 16. All blood samples were collected prior to immunizations.

The study was approved by the Norwegian Medicine Agency and the Norwegian South-Eastern Regional Committee for Medical and Health Research Ethics with written informed consent from all participants and monitored by Mericon (Skien, Norway).

2.2. T cell assays

2.2.1. Proliferation and IL-10 and TGF-β blocking assays

Peripheral-blood mononuclear cells (PBMC) were labelled with carboxyfluorescein succinimidyl ester (CFSE, Invitrogen Molecular Probes, OR, USA) as described earlier [9] before stimulation with Vacc-4x (5 µg/ml/peptide) along with unstimulated negative and positive controls (Staphylococcal enterotoxin B, 0.5 µg/ml, Sigma–Aldrich, MO, USA). In addition, parallel antigen-stimulated samples and controls cultures received blocking anti-IL-10 and anti-TGF-β monoclonal antibodies (mAbs) at 10 µg/ml, according to the instructions by the manufacturer (R&D Systems Europe, Abingdon, UK) and described elsewhere [17]. Cells were cultured in serum-free culture medium (Gibco AIM V, Invitrogen) with 0.5% highly purified human albumin for 6 days at 37 °C and 5% CO2 before harvesting, staining and preparation for flow cytometric analysis as previously detailed [9]. Cells were stained with anti-CD3 Pacific Blue, anti-CD8 AmCyan, anti-HLA-DR PE-Cy7 and 7-aminoactinomycin D (7-AAD, 7-aminocinomycin (7-AAD), the latter to exclude nonviable cells (Becton Dickinson Pharmingen, NJ, USA). Antigen-specific response was calculated as difference in percentage of proliferated (CFSE<sup>dim</sup>, activated (HLA-DR<sup>+<sup> and live (7-AAD<sup>−</sup>CD3<sup>+</sup>) T cell subsets (CD8<sup>+</sup> or CD8<sup>−</sup> defined as CD4<sup>+</sup>), respectively) between antigen-stimulated and control cultures, as previously detailed [14]. Multiple parallels were not due to shortage of available cells. IL-10- and TGF-β-mediated regulation of proliferation was estimated by subtracting antigen-induced proliferation from parallel controls with only IL-10- and TGF-β blocking mAbs (Fig. 1). Flow cytometry data were obtained with a BD Canto II with BD Diva software v6 and analyzed in WinList v7 (Verity Software House, ME, USA).

2.2.2. INF-γ and CD107a degranulation assays

Freshly isolated PBMC were stimulated with Vacc-4x (5 µg/ml/peptide) at 37 °C and 5% CO2 for 6h followed by harvesting, staining and preparation for flow cytometric analysis. Monensin (BD) and FITC-labelled anti-CD107a (eBioscience, CA, USA) were added prior to stimulation [18]. Two-step surface staining was performed with biotinylated anti-CD1-1 (R&D) and Streptavidin-APC (Invitrogen), the latter with anti-CD3 and anti-CD8, followed by permeabilization (PERMII, BD) and intracellular staining for interferon-γ (INF-γ) PE (BD). Cut-off for PD-1 was determined using the Fluorescence minus one method [19].

2.2.3. Granzyme B ELISPOT

The Granzyme B enzyme-linked immunosorbent spot (ELISPOT) assay was performed according to the instructions by the manufacturer (Mabtech, Sweden) with cryopreserved PBMC that were reconstituted overnight and stimulated in triplicate with Vacc-4x 15-mer overlapping peptides along with positive and negative controls using 200,000 PBMC/well. Spots were counted using an AID Elispot reader with AID Elispot v5 scanner software (AID GmbH, Germany) and median values of triplicates were used for analysis. Spot-forming units (SFU) were adjusted by the number of spots in negative controls. The proportion of CD8<sup>+</sup> T cells in each sample was enumerated by flow cytometry to calculate SFU per million CD8<sup>+</sup> T cells.
2.2.4. Soluble cytokine and chemokine assay

Cytokines and chemokines were measured in supernatants from cell cultures containing 200,000 PBMC/well from the proliferation assay after 24 h stimulation with Vacc-4x. IL-1β, IL-8, IL-10, IL-13, IL-17, IFN-γ-induced protein 10 (IP-10), monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1β (MIP-1β), RANTES and tumor necrosis factor-α (TNF-α) were measured using the BioPlex XMAP technology (TX, USA) with a Luminex IS100 instrument (BIO-RAD, CA, USA) and Bio-Plex manager Software v6. The StatLIA software package v3 (Brendan Scientific Inc., CA, USA) was used to calculate sample cytokine concentrations.

2.2.5. Delayed-type hypersensitivity testing (DTH)

Vacc-4x peptides were injected intradermally without GM-CSF at study end. The perpendicular diameters of palpable skin infiltrates were registered after 48 h as previously described [11,20].

2.3. Statistics

Responders and non-responders after the first (“primary”) or second (“overall”) booster were defined by positive or negative differences relative to baseline in vaccine peptide-specific CD8+ T cell responses, respectively. Mann–Whitney U (MWU), Kruskal–Wallis test, Spearman Rank, Wilcoxon signed-rank test and Fisher exact test were used to analyze differences between groups, correlations, dependent variables and cross-tabulated data, respectively (Statistics v7, StatSoft, OK, USA). Continuous variables are presented as median (interquartile ranges, IQR). A p-value <0.05 was considered significant.

3. Results

3.1. Safety and clinical data

Twenty-five of the 38 patients from the first Vacc-4x study [9] were available and eligible for re-immunization. After primary immunizations, all continued the 14 weeks per protocol interruption of ART for 1.8 years (median) and were thereafter treated with effective ART for 5.4 years (median) before inclusion in the present study. Clinical data are presented in Table 1.

After two Vacc-4x booster doses, only mild and transient discomfort was reported; 15 patients developed moderate local erythema or swelling at the injection site and five experienced additional muscle pain, fever, fatigue and headache after the first booster, whereas 18 patients reported local and three patients reported systemic symptoms after the second booster. No HIV-related complications were seen during the study period and all participants were asymptomatic at study end. Moreover, no changes were noted for median CD4 cell counts (587 vs. 582 cells/µL), CD8 cell counts (1227 vs. 1151 cells/µL) or HIV-RNA (<20 copies/ml).

3.2. Various patterns in Vacc-4x induced T cell responses following booster immunizations

Vacc-4x-specific in vitro responses were measured by T cell proliferation, CD8+ T cell degranulation (CD107a and Granzyme B) and IFN-γ production with rather large variations (Fig. 2). However, the data were consistent throughout the study with significant
correlations between the CD8+ and CD4+ T cell subsets as well as for the parameters for degranulation (data not shown).

Robust proliferative CD8+ T cell responses to Gag have consistently been related to slow progression of HIV [7,18]. Change in Vacc-4x proliferative CD8+ T cell responses relative to baseline was therefore chosen as the primary immunological read-out for boosting efficacy, in accordance with previous Vacc-4x studies [9].

At baseline, 19 (76%) patients had detectable proliferative CD8+ T cell responses to Vacc-4x (0.90% [0.28–1.32]). Twenty patients (80%) enhanced Vacc-4x CD8+ proliferation at some time point, either after the first (64%) or the second booster (16%). At the end of the study, 10 patients (40%) ended up as overall responders with permanently improved Vacc-4x CD8+ T cell proliferation relative to baseline (p = 0.005). The remaining 15 patients (60%) were overall non-responders, notably with decreasing Vacc-4x CD8+ T cell proliferation after two boosters (p = 0.001), with the same distribution of randomized Vacc-4x dose arms as overall responders (see [9]). However, quite different responses after the first booster were observed within each responder group (Fig. 3, right panels): for example, among overall responding patients, 5 patients achieved maximal proliferative responses after the first booster, but with a four-fold reduction in proliferation after the second (p = 0.046). In contrast, the 4 out of the 5 remaining overall responders tended to reduce responses after the first booster. Among the overall

\[
\begin{array}{|c|c|c|c|}
\hline
\text{Age (years)} & 51 (48–56) & 52 (49–56) & 50 (47–58) \\
\hline
\text{DTH (mm)}^a & 79 (39–154) & 143^b (104–398) & 51 (28–133) \\
\hline
\text{Years since HIV diagnosis} & 14.7 (13.0–19.4) & 15.6 (12.2–20.9) & 14.6 (13.6–19.4) \\
\hline
\text{Years on effective ART} & 5.4 (3.5–6.1) & 5.4 (3.3–6.1) & 4.8 (3.8–6.1) \\
\hline
\text{Nadir CD4 (cells/μl)} & 210 (150–230) & 205 (150–230) & 210 (150–270) \\
\hline
\text{CD4 count (cells/μl)} & 587 (435–815) & 702 (435–873) & 566 (423–687) \\
\hline
\text{CD8 count (cells/μl)} & 1227 (956–1571) & 1155 (813–1553) & 1233 (1026–1780) \\
\hline
\text{HIV RNA (copies/ml)} & <20 (<20–<20) & <20 (<20–<20) & <20 (<20–<20) \\
\hline
\end{array}
\]

Data presented as median (interquartile range).

\(^a\) DTH: delayed-type hypersensitive skin test (induration area) at week 200 of the initial Vacc-4x protocol [10,11].

\(^b\) p = 0.056 between overall responders and non-responders (Mann–Whitney U test).

![Proliferation](image1.png)

![IFN-γ](image2.png)

![CD107a](image3.png)

![Granzyme B](image4.png)

**Table 1**

Clinical data at inclusion.

**Fig. 2.** Vacc-4x-specific T cell responses at baseline (week 0), after the first (week 4) and second booster (week 16), measured by four different in vitro assays (CFSE\textsuperscript{+}/HLA-D\textsuperscript{R}-defined proliferation, CD107a, Granzyme B and INF-γ). Medians and interquartile range (IQR) indicated for CD8+ (●) and CD4+ (○) T cells, respectively.
non-responders, 10 patients improved Vacc-4x responses after the first booster whereas proliferation decreased in 5 (Fig. 3). Thus, across the overall response groups, a subgroup of patients seemed to profit from the first booster only, denoting them as primary responders, with the reservation that first and second responses were evaluated after different period of times relative to boosting.

At baseline, overall responders had higher frequencies of Vacc-4x-specific CD8⁺ T cell CD107a⁺ degranulation ($p=0.05$) and CD4⁺ T cell INF-γ production ($p=0.01$), whereas some overall non-responding patients had higher secretion of certain proinflammatory cytokines, but also IL-10 (see below). In addition, primary responders had higher baseline levels of Vacc-4x-specific degranulating (CD107a⁺) CD8⁺ T cells ($p=0.05$, data not shown). No differences in baseline proliferative response rates (70% and 80%, $p=0.46$), clinical parameters, HIV-RNA levels or CD4 cell counts were seen between overall responders and non-responders (Table 1).

Additional modalities of Vacc-4x induced CD8⁺ T cell responses after two boosters were also assessed and evaluated together. Nine (90%) overall responders improved at least two CD8⁺ T cell response modalities (mainly degranulation) and 40% of them even had improvements of more than two, which tended to be lower for overall non-responders (55% and 7%, respectively; $p<0.07$, Fisher).
3.3. Higher baseline levels of proinflammatory soluble factors in Vacc-4x overall non-responding patients

Cytokine and chemokine levels were measured in cell culture supernatants at weeks 0 and 16. At baseline, the overall proliferative CD8+ T cell response correlated both with Th1 (TNF-α; r = 0.55, p = 0.012) and Th2 cytokines (IL-13; r = 0.51, p = 0.02). Interestingly, the 5 overall non-responders who lost proliferative responses already after the first booster, had in fact the highest baseline levels of the proinflammatory factors TNF-α, IL-1β, MIP-1β and possibly also inhibitory IL-10 in response to in vitro stimulation with Vacc-4x peptides (Fig. 4). After two booster immunizations, overall non-responders increased Vacc-4x induced secretion of the Th2 cytokine IL-13 (p = 0.003, Wilcoxon) whereas overall responders increased secretion of MCP-1 (p = 0.009). Taken together, at baseline proinflammatory mediators, but also IL-10, were preferentially produced in those patients who did not profit on re-immunizations at all, while boosters particularly enhanced Vacc-4x-related production of the Th2 cytokine IL-13 in the same patients.

3.4. Delayed type hypersensitivity responses to Vacc-4x in vivo

Multiple DTHs were given during the initial Vacc-4x protocol [9] and in follow up studies. Most patients were anergic before primary immunization and developed maximal induration at study week 3 [10,11]. The last DTH before reboost was done at study week 200 of the initial Vacc-4x protocol, approximately three years before the current study. A total of 82% of the reboost patients demonstrated positive DTH >10 mm² at week 200 (79 mm² [39–154]) with possibly larger DTH in the overall responding reboost patients (Table 1).

In the present study, Vacc-4x DTH was again tested at end of study week 16, twelve weeks after the second booster. Twenty-two (96%) of the 23 patients tested had positive DTH (99 mm² [64–177]), still with possibly larger indurations in overall responders than non-responders (p = 0.056). The relevance of this simple in vivo test was supported by correlations with the last preceding Vacc-4x DTH (r = 0.57, p = 0.011), current CD8+ T cell proliferation (r = 0.56, p = 0.006) and increased production of IL-13 (r = 0.63, p = 0.003).

3.5. Increased IL-10 and TGF-β mediated downregulation of Vacc-4x T cell proliferation in overall non-responding patients

We next assessed the association between boosting, T cell proliferation and T cell regulation mediated by the key regulatory cytokines IL-10 and TGF-β as illustrated in Fig. 1. At baseline, in vitro downregulation of Vacc-4x-specific proliferation was similar in magnitude and prevalence between the CD8+ and CD4+ T cell subsets [16 (67%) and 15 (63%)] and between overall responders and non-responders. Notably, this parameter changed only in overall non-responders, who downregulated Vacc-4x-specific CD8+ T cell proliferation (p < 0.001, Wilcoxon). Consequently, overall non-responders demonstrated stronger in vitro IL-10 and TGF-β mediated downregulation of Vacc-4x-specific CD8+ T cell proliferation than overall responders after two boosters (p = 0.031).

The finding that two boosters either enhanced proliferation or induced downregulation of Vacc-4x proliferation was supported by an overall negative correlation between these two parameters (Fig. 5). Another inhibitory mechanism of effector T cells is expression of PD-1 [21,22]. Changes in the downregulation of Vacc-4x proliferation correlated positively to concurrent changes in the expression of PD-1 on Vacc-4x-specific CD8+CD107a+ T cells (Fig. 5).

4. Discussion

The objective of this study was to evaluate the effects of two Vacc-4x boosters on specific T cell immunity in previously
immunized HIV-infected subjects. Based on the substantial diversity in HIV-specific responses in general [18,23] and the variability we have seen in Vacc-4x responses [10], we expected differentiated responses to boosters and hypothesized a possible link to immune regulation.

In this cohort, most patients still had detectable Vacc-4x memory responses more than seven years after the primary immunization. The overall efficacy of two boosters was only 40% in terms of proliferative CD8+ T cell responses. However, 80% of the patients were responders at some time point, after either one or two boosters. Notably, only one patient was identified who responded positively to the first and then again to the second booster, and 20% progressively lost Vacc-4x responsiveness after each booster. Overall responders did not only improve CD8+ proliferation but also degranulative capacity, which is an important characteristic of effective cytotoxic effector T cells [24]. Re-immunization may therefore improve several Gag-specific cytotoxic CD8+ T cell response modalities in selected patients. Overall responders also demonstrated increased Vacc-4x-specific INF-γ production and CD107a degranulation, suggesting the induction of polyfunctional T cells. Another response parameter was DTH which related to clinical outcome after primary Vacc-4x immunizations [10,20]. In this study, DTH marginally improved compared to the last preceding DTH taken three years earlier and also correlated to CD8+ T cell proliferation.

Nevertheless, most patients (60%) ended up with Vacc-4x-specific proliferation below their baseline levels after two Vacc-4x boosters. To our knowledge, this is the first observation where unsuccessful boosting of a therapeutic HIV vaccine can be explained by increased IL-10 and TGF-β mediated downregulation of vaccine-specific T cell proliferation, in keeping with facilitation of therapeutic vaccination of mice obtained by IL-10 blockade in vivo [25]. Booster-induced changes in IL-10 and TGF-β mediated regulation were linked to enhanced expression of inhibitory PD-1 on Vacc-4x-specific effector cells. Thus, in vitro quantification of T cell regulation might be relevant when selecting patients for vaccine trials as well as for the individualization of booster regimens. For example, our data suggest that some of our current study patients should not have been boosted at all, and robust proliferative responses after the first booster apparently identified patients who in retrospect should not have been offered a second dose. However, further exploratory studies should include additional regulatory modalities, such as the functional regulation by PD-1 and CTLA-4 [26].

Several aspects of our study and exploratory variable for regulation need to be addressed. For example, use of cut-off thresholds to define “positive” or non-acceptance of “negative” antigen-specific responses in stimulated samples relative to control might masquerade as phenomenon. We therefore used the raw data throughout. We acknowledge that classification of responder based on minute differences in responses relative to baseline in some patients may be different by chance and altogether weaken the statistical analysis, but such cases should be randomly distributed. Moreover, the limited cohort size calls for confirmatory trials, but we were not able to recruit all previously vaccinated participants. In addition, regulation and activation might develop differently by comparing different peptide booster doses, which was fixed in this study, or with individualized time intervals between boosters. Since the Th2 cytokine IL-13 increased, it would also have been interesting to test antibody levels to the Vacc-4x peptides, which have been negligible in previous studies. It is also possible that booster-induced T cell regulation may develop differently with other vaccines or modes of immunization. Ideally, we also would have wanted some additional data: Firstly, future trials of therapeutic HIV vaccines with patients on ART should strive for ultrasensitive HIV RNA. However, this was here hampered mainly by insufficient plasma volume both from the current trial and previous follow-up of the cohort as well as lack of facilities in our region for this test. Secondly, characteristics of the cellular sources of IL-10 and TGF-β in each individual patient might have gained additional relevant information, although the combined actions of regulatory cytokines on T cell effector functions were in our opinion the most important outcome of this pilot study.

Conclusively, these findings show that Vacc-4x boosters variably improves or dampens T cell responses in different patients and that boosting should be individualized. Broad surveillance of T cell functions may help in this respect and assessment of vaccine-related immune regulation should be further explored as a potential new parameter.

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Contributions: Design of study and regulatory issues: DK, MS and IB; acquisition of data and monitoring: AL, KB, IB, JOH and HCA; analysis, interpretation and writing the paper: AL, KB, MS, BS, AMDR, DK
Conflicts of interest: Birger Sorensen is shareholder in Bionor Pharma and Maja Sommerfelt and Jens O. Holmberg were employed by the Company. Otherwise, there are no other conflicts of interest.

References


A Parameter for IL-10 and TGF-β Mediated Regulation of HIV-1 Specific T Cell Activation Provides Novel Information and Relates to Progression Markers

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Abstract

HIV replication is only partially controlled by HIV-specific activated effector T cells in chronic HIV infection and strategies are warranted to improve their efficacy. Chronic T cell activation is generally accompanied by regulation of antigen-specific T cell responses which may impair an effective control of chronic infections. The impact of HIV-induced T cell regulation on individual patients’ disease progression is largely unknown, since classical T cell activation assays reflect net activation with regulation as unknown contributing factor. We here explore a quantitative parameter for antigen-induced cytokine-mediated regulation (RAC) of HIV-specific effector T cell activation by functional antibody-blockade of IL-10 and transforming growth factor-β (TGF-β). HIV Env- and Gag-specific T cell activation and RAC were estimated in peripheral blood mononuclear cells from 30 treatment-naïve asymptomatic HIV-infected progressors (CD4 count ≥ 500/µl, HIV RNA 37500 copies/ml) stimulated with overlapping peptide panels for 6 days. RAC was estimated from differences in T cell activation between normal and blocked cultures, and related to annual CD4 loss, immune activation (CD38) and microbial translocation (plasma lipopolysaccharides). RAC was heterogeneously distributed between individual patients and the two HIV antigens. Notably, RAC did not correlate to corresponding classical activation. Env RAC correlated with CD38 and CD4 loss rates (r = 0.37, p = 0.046) whereas classical Gag activation tended to correlate with HIV RNA (r = −0.35, p = 0.06). 14 patients (47%) with low RAC’s to both Env and Gag had higher CD8 counts (p = 0.014) and trends towards lower annual CD4 loss (p = 0.056) and later start with antiretroviral treatment (p = 0.07) than the others. In contrast, patients with high RAC to both Env and Gag (n = 8) had higher annual CD4 loss (p = 0.034) and lower CD8 counts (p = 0.014). RAC to Env and Gag was not predicted by classical activation parameters and may thus provide additional information on HIV-specific immunity. RAC and other assessments of regulation deserve further in-depth exploration.

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Introduction

Chronic human immunodeficiency virus type 1 (HIV) infection leads to a variable but progressive loss of immune functions in most patients. The progression rate is mainly influenced by two opposing factors, namely HIV-associated chronic immune activation [1–3] and the efficacy of HIV-specific T cell responses [4,5]. Chronic immune activation expressed by CD38 on T cells, correlates strongly to disease progression and mortality [6–8]. It is partly sustained by enhanced systemic translocation of microbial products such as bacterial lipopolysaccharide (LPS) [9,10] and induces polyclonal B and T cell activation [11,12], accelerated T cell turnover [13,14] and immune exhaustion [2,15]. Effective viral control, on the other hand, seems to depend on the presence of polyfunctional HIV-specific CD8⁺ T cells [4].

A less clarified aspect of HIV-specific immunity is downregulation of the HIV-specific effector T cells, where regulatory T cells (Treg) play a central role [16,17]. Regulation of effector T cells protects the host from damage in chronic infection, but may also impair effective immune control. It is mediated by a number of mechanisms, including the expression of inhibitory receptors in the immune synapse such as CTLA-4 [18] and programmed death-1 (PD-1) [19,20], or via soluble inhibitory cytokines, particularly IL-10 and transforming growth factor-β (TGF-β). These two key inhibitory cytokines impede pro-inflammatory responses by T cells, natural killer cells, monocytes and macrophages and are secreted by a number of cell types including Treg [21–24].

The efficacy of T cell responses depends on the sum of stimulatory and regulatory signals. T cell regulation has been intensively studied, but with focus on single regulating mechanisms. However, how these various regulating mechanisms finally and in concert influence HIV-specific T effector cells and disease progression in individual patients has been little explored. This might be assessed for T cells in vitro by blocking downstream...
intracellular regulatory signal pathways during antigen stimulation. Recently we tested such an in vitro quantitive parameter for regulation in patients on antiretroviral treatment (ART) during reboost with a Gag peptide-based therapeutic HIV vaccine [25]. We estimated vaccine-specific cytokine-mediated regulation of CD8 T cell responses by blocking the effects of IL-10 and TGF (antigen-induced cytokine-mediated regulation, RAC). Notably, changes in RAC explained the substantial variations in booster efficacy, including cases where vaccine responses waned after each booster.

Since RAC seemed to reflect important features of HIV vaccine-specific T cell immunity during immunization, we hypothesized that the same parameter would also provide novel information in natural chronic HIV infection. In this study, we therefore compared RAC and activation of Gag- and Env-specific T effector cells in treatment-naïve patients. We found RAC to be heterogeneous, both between individual patients and between the two HIV antigens, and unfavourably related to HIV progression.

Materials and Methods

Patients

Thirty asymptomatic HIV-1 seropositive ART-naïve viremic progressors were included (23 males, 7 females). Their clinical characteristics are shown in Table 1. The patients represented a spectrum of HIV-associated immune activation determined by CD38 densities on total CD8 and CD8 PD-1 T cells [8] and were chosen from a larger cross-sectional prospective study on immunological factors in HIV. The study was approved by the Norwegian South-Eastern Regional Committee for Medical and Health Research Ethics. Informed consent was signed by each participant.

Activation Assays and Flow Cytometry

Peripheral-blood mononuclear cells (PBMC) were isolated using Cell Preparation Tubes (Becton Dickinson (BD), CA, USA) and preserved, thawed and cultured in serum-free AIM culture medium containing 0.5% human albumin at 5% CO2 at 37°C, as described elsewhere [26]. To evaluate activation and proliferation parameters, the fractions of T cells co-expressing CD25 and HLA-DR [27] or having low carboxyfluorescein succinimidyl ester (CFSE) signal [28] were compared. PBMC were pulse-labelled with CFSE (3 μM, 5 min (Invitrogen Molecular Probes, OR, USA) as detailed previously [29] and subjected to HIV antigens (four HIV-1 Gag p24 consensus peptide sequences, represented by 15-mer overlapping by 2 amino acid panels [29] and non-HIV antigens (23 15-mer peptides from cytomegalovirus, Epstein-Barr virus and influenza virus (CEF, Mabtech, Sweden)).

For the estimates of antigen-specific cytokine-mediated regulation of T cell activation (RAC) cryopreserved PBMC were thawed, washed and reconstituted in serum-free AIM overnight, and then stimulated with complete 15-mer Env or Gag overlapping peptide panels (NIH AIDS Research and Reference Reagent Program, MD, USA) as detailed elsewhere [30]. Peptide panels in all experiments were used at 2 μg/ml/peptide. Peptide-exposed and control cultures were in parallel incubated with inhibitory monoclonal antibodies (mAbs) to IL-10 and TGF-β, each at 10 μg/ml final concentration according to the instructions by the manufacturer (R&D Systems Europe, Abingdon, UK), a concentration that abolished IL-10 in cell culture supernatants (Luminex assay of supernatants from antigen stimulated T cells cultured for 6 days, data not shown). Staphylococcal enterotoxin B (Sigma-Aldrich, MO, USA) was used as positive control at 0.5 μg/ml.

Cells were cultured at 37°C in 5% CO2 for 6 days, and then harvested, stained and prepared for flow cytometric analysis as previously described [31]. The following fluorochrome-labelled mAbs were used: CD3 Pacific Blue, CD8 AmCyan, HLA-DR PE-Cy7 (BD), CD4 PE and CD25 APC (eBioscience, CA, USA). 7-aminoactinomycin (7-AAD, BD) was added to discriminate between viable and non-viable cells according to the manufacturer. Flow cytometry data were obtained with a BD FACS Canto II with BD Diva software v6.1. Only lymphocyte and lymphoblast gates containing live 7-AAD- CD35 T lymphocytes were evaluated.

Table 1. Cohort characteristics.

<table>
<thead>
<tr>
<th>All (n = 30)</th>
<th>Median (IQR) range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>42 (33–49)</td>
</tr>
<tr>
<td>Time HIV seropositive (months)</td>
<td>57 (16–83)</td>
</tr>
<tr>
<td>CD4+ T cell count (x10⁶/l)</td>
<td>472 (325–695)</td>
</tr>
<tr>
<td>CD8+ T cell count (x10⁶/l)</td>
<td>1084 (788–1828)</td>
</tr>
<tr>
<td>HIV-RNA in plasma (copies/ml)</td>
<td>37500 (2300–72000)</td>
</tr>
<tr>
<td>Annual CD4 T cell count loss (cells ×10⁶/l)</td>
<td>11 (-69–177)</td>
</tr>
<tr>
<td>Îµ-microglobulin in serum (mg/l)</td>
<td>2.5 (1.9–3.3)</td>
</tr>
<tr>
<td>CD38 on CD8 T cells (molecules/cell)</td>
<td>3285 (1834–7226)</td>
</tr>
<tr>
<td>CD38 on CD8+CD38+PD-1+ T cells (molecules/cell)</td>
<td>4127 (2095–8704)</td>
</tr>
<tr>
<td>LPS (pg/ml)</td>
<td>70 (59–86)</td>
</tr>
</tbody>
</table>

doi:10.1371/journal.pone.0085604.t001

Quantification of Env and Gag Related T Cell Activation

Antigen-specific activation of T cell subsets was defined as the difference in activation marker between peptide-stimulated cells and corresponding control cells without peptides. In preceding experiments exploring T cell regulation by HIV vaccine antigens in patients on ART, regulation and activation were determined by differences in proliferation (CFSEmedian) in CFSE pulse-labelled cells [25]. In our experience, thawed PBMC samples from ART-naïve individuals are more vulnerable to toxic effects of CFSE [32], even after short exposure and low concentrations. We therefore compared fractions of CFSEmedian; defined by median fluorescence intensities equal to or below the second proliferated generation of CFSE-labelled PBMC, and fractions of CD25+HLA-DR. These parameters reflect overlapping aspects of T cell activation [27], i.e. proliferation, IL-2 receptor expression and increased HLA class II expression), as illustrated in Fig. 1A, with correlating activation results after exposure to both non-HIV and HIV antigens (Fig. 1B). T cell activation within the cohort was therefore determined by the frequency of subsets co-expressing CD25 and HLA-DR in antigen-stimulated cultures corrected for unstimulated controls [27].

Quantification of Env- and Gag-induced Cytokine-mediated T Cell Regulation

In parallel with classical activation cultures, IL-10 and TGF-β blocked activation was determined as the difference between antigen-stimulated and control samples that received IL-10 and TGF-β blocking mAbs (Fig. 2A). The magnitude of antigen-induced IL-10 and TGF-β mediated regulation of T cell activation (RAC) was calculated by the difference in activation between these two culture conditions (Fig. 2B). RAC calculated by CFSEmedian
correlated with $R_{\text{MC}}$ determined by the CD25$^+$HLA-DR$^+$ subsets (Fig. 2C).

LPS Measurement

EDTA plasma obtained concurrently with the isolation of PBMC was separated and snap-frozen at $-70^\circ$C. LPS was later measured en bloc in thawed plasma with the Limulus Amebocyte Lysate chromogenic assay (Lonza, MD, USA) according to the manufacturer’s instructions with the following modifications: Samples were diluted 10-fold to avoid interference with background colour and preheated to $70^\circ$C for 12 minutes prior to analysis to dissolve immune complexes, as previously described [10].

**Figure 1. Comparison of CD25$^+$HLA-DR$^+$ expression and CFSE$^{\text{dim}}$ as measures for antigen-induced activation and regulation of T cells.** CFSE-labelled PBMC from 28 HIV-infected individuals were stimulated for 6 days with peptides encoding either HIV p24 consensus regions (HIV Ag) or a pool of commonly encountered non-HIV viral peptides (Non-HIV Ag). A. Co-expression of CD25 and HLA-DR on live CD8$^+$CD3$^+$ T cells on non-divided CFSE$^{\text{high}}$ (left panel) and proliferated CFSE$^{\text{dim}}$ T cells (right panel), showing excessive difference in fractions of CD25$^+$HLA-DR$^+$ in the activated subset to the right. B. Scatter plots of activation measured in the same culture by CFSE$^{\text{dim}}$ or HLA-DR$^+$CD25$^+$, respectively, to non-HIV (□) and HIV antigen (○) within the CD8$^+$ (left panel) and CD4$^+$ (right panel) T cell subsets. Significant and high correlations obtained for both Non-HIV antigens (CD8$^+$, $r = 0.92$, $p < 0.001$; CD4$^+$ $r = 0.64$, $p < 0.001$) and HIV Gag p24 (CD8$^+$, $r = 0.90$, $p < 0.001$; CD4$^+$ $r = 0.71$, $p < 0.001$).

doi:10.1371/journal.pone.0085604.g001

**Plasma Levels of Cytokines and Chemokines**

Soluble cytokines representing Th1 (TNF-$\alpha$, INF-$\gamma$) and Th2 (IL-4, IL-5, IL-10 and IL-13) profiles were measured in snap-frozen EDTA plasma (see above) using Bio-Plex XMap technology (TX, USA) with a Luminex IS100 instrument (BIO-RAD, CA, USA) and Bio-Plex manager Software v6, according to the instructions by the manufacturer.

**Statistics**

To not underestimate regulation, antigen-specific activation readouts relative to control cultures were treated as raw data. Non-parametrical statistics were applied throughout the study; Mann-Whitney U- and Kruskal-Wallis test to compare differences between two or more groups, and Spearman Rank for correlation analysis. All continuous variables are presented as medians.
Results

Cohort Characteristics Including Parameters for Immune Activation

Thirty asymptomatic ART-naïve HIV-infected patients (CD4+ T cell counts, 472; HIV RNA, 37,500 copies/ml, medians) were included to represent a spectrum of HIV-associated immune activation. CD38, microbial translocation (LPS) and HIV RNA correlated (r between 0.44–0.60, p<0.02, detailed data not shown). In keeping with previous observations where CD38 density on CD8+ T cells and on CD8+PD-1+ cells had higher correlation with other progression markers than frequencies of CD38+HLA-DR+CD8+ T cells [8,25,26], CD38 density was used to represent chronic immune activation in the following analysis.

T cell Activation by Gag and Env

T cell activation to Gag and Env peptide panels varied between patients and was generally higher for Gag, in keeping with previous observations [30] (Fig. 3A, x-axis). Moreover, Gag and Env activation correlated within both the CD8+ (r = 0.40, p = 0.027) and CD4+ (r = 0.53, p = 0.003) T cell subsets (data not shown).

Variable T Cell Regulation (R_{AC}) without Correlation to Activation

A parameter for HIV antigen-specific cytokine-mediated T cell regulation (R_{AC}) was determined by parallel antigen activation cultures and controls in the absence and presence of IL-10 and TGF-II blocking mAbs. It should be noted that R_{AC} calculated by CFSE correlated significantly with R_{AC} determined by the co-expression of CD25 and HLA-DR (Fig. 2C).

A substantial variability was observed in R_{AC} related to Gag and Env exposure (Fig. 3A, y-axis). No correlations were found between R_{AC} induced by the two HIV antigens (Fig. 3B), in contrast to the corresponding activation. Perhaps more importantly, Gag or Env related R_{AC} and corresponding activation did not correlate (Fig. 3A). Thus, R_{AC} quantified this way could not have been predicted by the conventional activation assay.

Activation and R_{AC} to HIV-antigens in Relation to Progression Markers

We next explored how R_{AC} was related to markers of chronic HIV activation (CD38 density on CD8+ T cells and PD-1 subsets [8,26]), microbial translocation (LPS), HIV replication and annual CD4+ T cell loss rates. Significant and unfavourable correlations were revealed between Env related R_{AC} in either T cell subsets and chronic immune activation (CD8+, r = 0.41, p = 0.024) and CD4 loss rates (CD4+, r = 0.39, p = 0.032), whereas Gag-induced T cell activation tended to correlate with HIV RNA (CD8+, r = -0.35, p = 0.060). These heterogeneous relations are depicted in Fig. 4, for simplicity illustrated by overall CD3+ T cell activation and regulation.

Clusters of Patients with Low and High HIV Antigen-induced Regulation

One cluster of patients appeared to have low R_{AC} induced by both Gag and Env within the CD4+ and CD8+ subsets (Fig. 3B). The same cluster was seen when we examined R_{AC} for all CD3+ T cells (Fig. 4A). This is in keeping with the notion that IL-10 and
TGF-β inhibit both the CD4+ and CD8+ T cell subsets \[23,33\]. This cluster of patients with overall low RAC induced by Gag and Env was defined as Low regulators \(n = 14\) (47%) \(\text{Fig. 5A}\) whereas the remaining 53% \(n = 16\) were termed High regulators. Notably, the magnitude of RAC in suppressing corresponding activation was quite substantial for the High regulator patients, as illustrated by high RAC/Activation-ratios \[3.0 (0.8–4.1)\] for Env and \[2.4 (0.8–12.4)\] for Gag, respectively \(\text{data not shown}\). Again, conventional activation for CD3+ T cells did not correlate with the corresponding RAC. Thus, High regulators could not have been identified by the activation assay \(\text{Fig. 5B}\).

The Low and High regulator patient groups were also compared with respect to clinical parameters, immune activation, LPS and conventional activation. High regulators had lower CD8 counts in blood \(p = 0.031\) and a trend towards faster CD4 loss rates \(p = 0.056\) \(\text{Table 2}\). High regulators also had significantly lower levels of plasma Th1 cytokines INF-γ \(p = 0.04\) and TNF-α \(p = 0.04\) \(\text{Fig. 6}\), but no differences were found for Th2 cytokines including IL-10 between the two regulation groups.

Characterization of Study Patients with High HIV Antigen-induced Regulation

Examining the High regulator patients in more detail, we found that they either had substantial RAC induced by Gag \(\text{denoted Gag regulators, } n = 8\) \(\text{Fig. 5A}\), or by both Gag and Env \(\text{Pan regulators, } n = 8\) \(\text{Fig. 5A}\). Gag regulators appeared more similar to Low regulators in most parameters except that they had less conventional activation to both Gag (CD4+ subset, \(p = 0.016\)) and Env \(\text{p = 0.025}\). Pan regulators, on the other hand, had a profile compatible with more accelerated disease, such as higher annual CD4 loss \(221 \text{ vs } 210 \text{ cells/year, } p = 0.034\), lower CD8 counts \(\text{median 841 vs 1458 cells/μl, } p = 0.014\) and possibly lower CD4 counts \(\text{median 254 vs 488 cells/μl, } p = 0.065\) compared with the Low regulator patients \(\text{Fig. 6}\). Thus, one might speculate whether Gag and Pan regulators represent a continuum of an unfavourable regulator phenotype which could not be identified by the classical activation assay. Finally, of the 14 patients who had started ART according to current guidelines within one year post-inclusion, more patients tended to be Pan regulators than belonging to the other subgroups \(6 \text{ of 8 vs. } 8 \text{ of 22, } p = 0.07\).
Discussion

HIV-specific T effector cells are potentially able to control viral replication in HIV infection, but their responses are critically weakened by the initial loss of HIV-specific CD4$^+$ T cells, viral immune escape, and T cell exhaustion driven by immune activation [5,34]. An additional counteracting factor might be the regulation of effective HIV specific T effector cells. We here assessed a functional quantitative parameter for T cell regulation ($R_{AC}$) which we think could be relevant when evaluating HIV infected patients and developing therapeutic vaccines. Therapeutic vaccines might play an essential role in a future cure for HIV by inducing effective T cell responses against re-activated, latently infected cells [34]. Theoretically, pre-existing or induced regulation can evoke T cell anergy and thus hamper the effects of therapeutic vaccination in some patients. This notion was supported by our recent observation where changes in $R_{AC}$ explained variable and in some cases negative responses to therapeutic HIV vaccine boosters [25].

To our knowledge, this is the first attempt to determine $R_{AC}$ or similar quantitative parameters for HIV antigen-specific regulation in chronically infected treatment-naïve patients. The study was motivated by our expectation that $R_{AC}$ would provide

![Figure 4. Relations between Env- and Gag-induced $R_{AC}$ and activation and markers related to progression of chronic HIV infection.](doi:10.1371/journal.pone.0085604.g004)

$R_{AC}$ and corresponding activation (x-axis), for simplicity represented as overall CD3+ estimates (CD4$^+$ plus CD8$^+$ combined) induced by Env and Gag peptide panels, respectively, and in relations to various progression markers (y-axis). Spearman rank r and corresponding p-values indicated, values with p<0.10 bolded.

A Parameter for HIV-1 T Cell Regulation

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additional prognostic information. We found considerable variability in \( R_{\text{AC}} \) not only between individual patients, but also between the two tested HIV antigens. Thus, our data suggest that at least in some patients, \( R_{\text{AC}} \) does not reflect "global" regulation of HIV antigens. \( R_{\text{AC}} \) was in some cases substantial, exceeding activation more than ten-fold. Moreover, \( R_{\text{AC}} \) did not relate to corresponding conventional activation readouts, showing that it provided additional otherwise hidden information.

This exploratory approach to characterize a parameter apparently reflected at least some aspects of cytokine-mediated regulatory "capacity" in the individual patient. However, although our data suggest that \( R_{\text{AC}} \) can differentiate HIV-infected patients in a new way and may reflect processes that are related to progression of HIV, our choices of assay read-out and culture conditions need to be commented: Several assays are frequently used to assess HIV-specific T cell activation and function. For example, polyfunctional T cells in 6 to 18 h cultures have been shown to coincide with control of viral replication [32]. However, we did not prioritize this assay due to shortage of cells from this clinically well-defined cohort, and 6 day cultures were chosen for several reasons: First, we expected a \textit{priors} that antigen-related regulation is a slower, secondary response, to primary activation. This assumption is in keeping with the observation that stimulation of resting Treg reach maximal expression of FoxP3 34–44 h after simulation [35]. Second, it is still not clear whether early polyclonality actually persists over time, including early markers for proliferation such as Ki-67 [32]. Third, a fundamental element of effector lymphocytes is the ability to proliferate, indicating responsiveness to IL-2 (via its receptor CD25), whereas proinflammatory cytokines such as IFN-\( \gamma \) upregulate HLA class II (including DR) on T cells. Moreover, HIV-specific proliferative T cell responses have been long known to associate with slow progression [36]. Our assay use changes in CD25+HLA-DR+ as readout, parameters that both reflect activation and proliferation, the latter illustrated in Fig. 1A. Nevertheless, we appreciate that our approach only reflect one out of several ways by which classical "net" T cell responses can be estimated \textit{in vitro}. Indeed, other major regulatory pathways may influence overall activation. Finally, in-depth interpretation and characterization of our assay can certainly be extended, such as to address whether the "gain" in activation by blockade of regulatory pathways also provides an increase in effector cell functions, such as cytotoxic capacity or polyfunctionality.

A possible clinical relevance of this new exploratory parameter was suggested by the significant correlations between \( R_{\text{AC}} \) and the classical prognostic markers CD38 and CD4 loss rates. These correlations were not found for the activation results (Fig. 4). Even if the study included only a limited number of cases, we were still able to cover a wide spectrum of chronic immune activation. Gag-specific T cell responses correlated negatively with concurrent HIV RNA levels, an association also found in other and larger study cohorts [37,38]. It should be noted that our group favours bead-calibrated measures for CD38 density rather than the more simple and conventional measure for HIV-associated chronic immune activation, namely % CD38+HLA-DR+. We have previously shown that CD38 density is even better related to other progression markers [8,25,26].

Post-hoc we observed clusters of patients having either particularly low (Low regulators) or high (High regulators) regulation (i.e. \( R_{\text{AC}} \)). The High regulators seemed to have more rapid HIV progression, in keeping with our expectation. In contrast, Low regulators had more favourable clinical characteristics in terms of slower CD4 loss rates and higher CD8 counts [39]. The levels of the proinflammatory cytokines TNF-\( \alpha \) and IFN-\( \gamma \) were also higher in Low regulators. This has previously been interpreted as a sign of unfavourable immune activation in patients with lower CD4 counts [40,41]. From our data, derived from patients with higher CD4 counts, one might conversely speculate whether higher TNF-\( \alpha \) and IFN-\( \gamma \) levels rather reflect a beneficial type of immune activation.

\( R_{\text{AC}} \) or similar quantitative parameters for HIV antigen-specific regulation should be further explored in larger cohorts. This may help to better understand the complex interplay between regulation and activation, to select patients for immune therapy.
studies, and to determine the prognostic significance of regulation. Future studies should also explore the individual contribution of IL-10 and TGF-β along with other regulating mechanisms such as CTLA-4 and PD-1. This was hampered by a scarcity of patients and samples in this study. Both a broader range of HIV antigens and even non-HIV antigens should be tested. In this study Gag was selected based on the relation between Gag-specific T cell responses to control viral replication [37,38] and Env as a relevant antigen for HIV vaccines.

Figure 6. Distributions between regulator subgroups. Box and whisker plots representing medians, interquartile ranges and overall ranges for cytokines in snap-frozen plasma (upper two panels), CD4 counts and CD4 loss rates (two middle panels) and CD8 counts as well as HIV RNA levels (two lower panels). “Low regulators” (Low) as defined in the text are represented as one group, whereas the “High regulator” patients are split into “Gag regulators” (Gag) and “Pan regulators” (Pan), respectively. Significant differences p<0.05 between groups (Mann-Whitney) indicated (*). doi:10.1371/journal.pone.0085604.g006
Table 2. Characteristics of regulator groups.

<table>
<thead>
<tr>
<th></th>
<th>Low regulators (n = 14)</th>
<th>High regulators (n = 16)</th>
<th>Low vs. high regulators</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median (IQ range)</td>
<td>Median (IQ range)</td>
<td>p*</td>
</tr>
<tr>
<td>Age (years)</td>
<td>43 (39–49)</td>
<td>40 (30–52)</td>
<td>0.755</td>
</tr>
<tr>
<td>Time HIV seropositive (months)</td>
<td>65 (16–100)</td>
<td>52 (21–68)</td>
<td>0.510</td>
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<tr>
<td>CD4+ T cell count (×10^6/l)</td>
<td>488 (438–565)</td>
<td>392 (225–825)</td>
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<tr>
<td>CD8+ T cell count (×10^6/l)</td>
<td>1458 (880–2148)</td>
<td>938 (663–1261)</td>
<td>0.031</td>
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<td>HIV-RNA in plasma (copies/ml)</td>
<td>17000 (670–52000)</td>
<td>43000 (4000–135000)</td>
<td>0.220</td>
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<tr>
<td>Annual CD4 T cell count loss (cells ×10^6/l)</td>
<td>−10 (−75–41)</td>
<td>62 (−46–235)</td>
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<td>β2-microglobulin in serum (mg/l)</td>
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<td>2.3 (1.5–5.3)</td>
<td>0.965</td>
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<td>CD38 on CD8+ T cells (molecules/cell)</td>
<td>1894 (1770–7116)</td>
<td>4271 (1688–11165)</td>
<td>0.244</td>
</tr>
<tr>
<td>CD38 on CD8+CD38+PD-1 + T cells (molecules/cell)</td>
<td>2541 (1938–8101)</td>
<td>4946 (1962–13798)</td>
<td>0.228</td>
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<tr>
<td>LPS (pg/ml)</td>
<td>70 (57–76)</td>
<td>70 (59–92)</td>
<td>0.693</td>
</tr>
</tbody>
</table>

*Comparisons between patient groups, p < 0.05 bolded, p < 0.10 italic. doi:10.1371/journal.pone.0085604.t002

Conclusions

In summary, this study on regulation of Gag- and Env-specific T cell activation by IL-10 and TGF-B (R_g) in chronic HIV infection revealed heterogeneous levels of regulation between both patients and HIV antigens. The magnitude of R_g was substantial in some individuals and R_g could not be predicted by the corresponding, classical antigen-specific activation parameters. High R_g seemed clinically unfavourable, particularly when induced by Env peptides. Thus, assessments of regulation deserve further in-depth exploration and extension to larger cohorts.

References


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Author Contributions

Conceived and designed the experiments: AL, DK. Performed the experiments: AL, KB, TEM, MT. Analyzed the data: AL, KB, FOP, DK. Contributed reagents/materials/analysis tools: AL, KB, FOP TEM MT DK. Wrote the paper: AL, KB, DK.

Author Contributions

Conceived and designed the experiments: AL, DK. Performed the experiments: AL, KB, TEM, MT. Analyzed the data: AL, KB, FOP, DK. Contributed reagents/materials/analysis tools: AL, KB, FOP TEM, MT DK. Wrote the paper: AL, KB, DK.


