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MULTIDISCIPLINARY ANALYSIS OF HIV-1 ELITE CONTROLLERS

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Multidisciplinary analysis of HIV-1 Elite Controllers

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ABSTRACT

Chronic HIV-1 infection is characterized by progressive depletion of CD4+ T lymphocytes, persistent immune activation and ongoing viral replication, leading to a profound immunodeficiency state if left untreated with antiretroviral therapy. However, a small percentage of infected individuals are able to maintain durable control of HIV replication and stable CD4 counts, in the absence of antiretroviral treatment (ART). This rare group of individuals are known as Elite Controllers (ECs) and represent evidence that control of infection without ART for years is possible, thereby providing an extraordinary insight into new vaccine and functional cure strategies. Despite extensive studies, the specific mechanisms by which ECs maintain control remain undefined. A better understanding of host factors that contribute to how ECs spontaneously control the infection is crucial for future therapeutic strategies.

In **Paper I**, we showed that ECs possessed a richer gut microbiota compared to untreated HIV-infected individuals, and that several metabolic pathways were significantly different to untreated individuals. Specifically, the tryptophan catabolism pathway in ECs was very similar to healthy subjects, indicating a contributing factor for lower persistent immune activation usually observed in HIV-infected individuals. Our data suggest that the unique bacterial composition and metabolic profile of ECs may be involved in control of infection. Further, in **Paper II**, we used a modified antibody assay, *LIPS*, to perform antibody profiling against HIV-1 proteome in ECs. We found that *LIPS* detected a strong response against several HIV-1 fusion proteins in ECs compared to long-term treated individuals. Interestingly, the observed heterogeneity in antibody levels among ECs were not very different from untreated, viremic patients, indicating a non-homogenous patient group among ECs and a continuous viral expression with limited release of virus.

By adapting a comprehensive analysis strategy of transcriptomics and targeted proteomics (**Paper III**), we demonstrated that more than 150 protein-coding genes and 33 soluble factors were differentially expressed in ECs compared to untreated patients. In particular, CXCR6 and SIGLEC1 (associated with viral entry and formation) were downregulated in ECs. Also, PD-1, an inhibitory receptor associated with T cell exhaustion, was significantly elevated in untreated vs both ECs and healthy subjects. The observed difference between ECs and untreated patients in molecular pathways regulating apoptosis, inflammation and cellular differentiation, suggests they play a synergistic role in HIV control. To further understand the differences in inhibitory receptor expression related to spontaneous HIV control, we assessed the expression of inhibitory molecules associated with T cell exhaustion on CD4+ T cells (**Paper IV**). We observed that ECs maintain a co-expression pattern of inhibitory receptors similar to healthy subjects and significantly different to both treated and untreated patients. We found that ECs harbor a “healthy” state of inhibitory receptor expression on CD4+ T cells that might play part in maintenance of their control status.

In summary, this thesis describes a comprehensive analysis of important immune factors that is associated with natural control of HIV infection in ECs. The multidisciplinary approach has provided a better understanding for the complexity of spontaneous HIV control and possible future therapeutic interventions.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Infektion med humant immunbristvirus (hiv) har orsakat otroliga katastrofer under de senaste 40 åren världen har känt till viruset. Infektionen och dess utveckling av sjukdomen AIDS har kostat flera miljoner människor deras liv och idag lever närmare 37 miljoner med hiv. Det finns effektiva bromsmediciner som möjliggör att en infekterad person kan leva ett relativt bra liv, men lång ifrån alla har tillgång till dessa mediciner. Det behövs ett botemedel mot hiv och enorma insatser har gjorts i försök att hitta ett vaccin som skyddar mot hiv, dock utan framgång. Det finns en väldigt liten grupp hiv-infekterade personer som lyckas hålla hivinfektionen i schack, dvs de har omätbara virus nivåer och deras immunförsvar mår relativt bra, utan att de behöver bromsmediciner. Kort sagt, dessa unika *elite kontroller* är ett bevis på att man kan kontrollera infektionen utan mediciner och inte utvecklar AIDS. Studerandet av elite kontroller kan ge en otrolig insyn på vilka underliggande biologiska mekanismer som ger upphov till denna naturliga kontroll av infektion. Denna avhandling hade som syfte att försöka förstå den immunologiska aspekten av kontroll, detta genom att studera T celler, antikroppsnivåer, tarmfloran och immunaktivering samt gen- och protein uttrycket hos 19 identifierade elite kontroller i Sverige. I **studie I** fann vi att elite kontroller har en rik tarmflora med en unik bakteriesignatur som skiljde sig avsevärt från andra hivinfekterade, men var väldigt lik icke-infekterade (”friska”) personer. Olika bakteriegrupper var antingen mer frekventa eller nästan obefintliga hos elite kontroller, och dessa bakteriegrupperna hade ett statistik samband med immunaktivering. Vi såg även att ämnesomsättningsprofilen skiljde sig från elite kontroller och friska samt hivinfekterade, vilket tyder på att tarmfloran spelar en viktig roll i kontrollen av viruset. Vidare undersökte vi antikroppsnivåer mot olika hivproteiner hos elite kontroller (**studie II**) och fann att nivåerna varierade avsevärt bland dem. Nivåerna var högre hos elite kontrollerna än hos behandlade hivpatienter och lika de som är obehandlade med höga virusnivåer. Detta tyder på en underliggande, lågt puttrande virusreplikation samt heterogenitet bland elite controller. I **studie III** tillämpades en övergripande analysstrategi för att undersöka gen- och proteinuttrycket. Mer än 150 proteinkodande gener och 33 lösliga komponenter av immunsystemet var uttryckt annorlunda hos elite kontroller än hos obehandlade hivpatienter. Specifikt CXCR6 och SIGLEC1, som är molekyler associerade med hur hiv tränger in i cellen och cellformation, var nedreglerad hos elite kontroller. De signifikanta skillnaderna i molekylära processer som reglerar celldöd, inflammation och celldifferentiering tyder på att de samspelar för att behålla viruskontrollen i dessa individer. Slutligen så undersökte vi konceptet med ”T cells utmattning” genom att mäta uttrycket av inhibitoriska receptorer på CD4+ T cellerna (**studie IV**) och fann att uttrycket av TIGIT, CTLA-4 och PD-1 var lika mellan elite kontroller och friska individer, och olika gentemot välbehandlade och obehandlade hivpatienter. Elite kontroller har en ”frisk repertoar” av CD4+ T celler som kan spela en roll i att hålla viruset i schack. Sammanfattningsvis så visar studierna i denna avhandling att elite kontroller har flera olika parametrar som definierar deras kontroll status och att de mycket troligtvis samspelar i denna kontroll. Att fortsätta studera dessa unika elite kontroller är av ytterst vikt för att i framtiden kunna översätta resultat till ett eventuellt vaccin mot hiv.

LIST OF SCIENTIFIC PAPERS

I. Jan Vesterbacka, Javier Rivera, **Kajsa Noyan**, Mariona Parera, Ujjwal Neogi, Malu Calle, Roger Paredes, Anders Sönnerborg, Marc Noguera-Julian, Piotr Nowak. *Richer gut microbiota with distinct metabolic profile in HIV infected elite controllers*. Sci Rep. 2017, Jul 24;7(1):6269.

II. Wang Zhang, Mohammed M. Morshed, **Kajsa Noyan**, Aman Russom, Anders Sönnerborg, Ujjwal Neogi. *Quantitative humoral profiling of the HIV-1 proteome in elite controllers and patients with very long-term efficient antiretroviral therapy*. Sci Rep. 2017 Apr 6;7(1):666

III. Wang Zhang, Anoop T. Ambikan, Maike Sperk, Robert van Domselaar, Piotr Nowak, **Kajsa Noyan**, Aman Russom, Anders Sönnerborg, Ujjwal Neogi. *Transcriptomics and targeted proteomics analysis to gain insights into the immune-control mechanisms of HIV-1 infected elite controllers*. EBioMedicine. 2018 Jan;27:40-50.

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I. Marcus Buggert, Son Nguyen, Laura M. McLane, Maria Steblyanko, Nadia Anikeeva, Dominic Paquin-Proulx, Perla M. Del Rio Estrada, Yuria Ablanedo-Terrazas, **Kajsa Noyan**, Morgan A. Reuter, Korey Demers, Johan K. Sandberg, Michael A. Eller, Hendrik Streeck, Marianne Jansson, Piotr Nowak, Anders Sönnerborg, David H. Canaday, Ali Naji, E. John Wherry, Merlin L. Robb, Steven G. Deeks, Gustavo Reyes-Teran, Yuri Sykulev, Annika C. Karlsson, Michael R. Betts. *Limited immune surveillance in lymphoid tissue by cytolytic CD4+ T cells during health and HIV disease.* PLoS Pathog. 2018 Apr 13;14(4):e1006973.

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IV. Jenny Svärd, Dominic Paquin-Proulx, Marcus Buggert, **Kajsa Noyan**, Babilonia Barqasho, Anders Sönnerborg, Piotr Nowak. *Role of translocated bacterial flagellin in monocyte activation among individuals with chronic HIV-1 infection*. Clin Immunol. 2015 Dec;161(2):180-9.

V. Marcus Buggert, Juliet Frederiksen, **Kajsa Noyan**, Jenny Svärd, Babilonia Barqasho, Anders Sönnerborg, Ole Lund, Piotr Nowak, Annika C. Karlsson. *Multiparametric Bioinformatics Distinguish the CD4/CD8 ratio as a suitable laboratory predictor of combined T cell pathogenesis in HIV infection*. J Immunol. 2014 Mar 1;192(5):2099-108.

CONTENTS

LIST OF ABBREVIATIONS

Abs	Antibodies
ADCC	Antibody-dependent cell-mediated cytotoxicity
AIDS	Acquired immune deficiency syndrome
ART	Antiretroviral treatment
bNAbs	Broadly neutralizing antibodies
CASCADE	The Concerted Action on SeroConversion on AIDS and Death in Europe collaboration
CCR	CC chemokine receptor
CD	Cluster of differentiation
CTL	Cytotoxic T cell
CXCR	CXC chemokine receptor
DCs	Dendritic cells
ECs	Elite controllers
Env	Envelope protein
Gag	Group-specific antigen
GALT	Gut associated lymphatic tissue
GI tract	Gastrointestinal tract
gp	Glycoprotein
HDAC	Histone deacetylase inhibitors
HIV	Human immunodeficiency virus type 1
HLA	Human leukocyte antigen
HTLV	Human T-cell leukemia virus
IDO	Indoleamine 2,3-dioxygenase-1
IFN	Interferon
IL	Interleukin
KIRs	Killer cell immunoglobulin-like receptors
LIPS	Luciferase immuno-precipitation systems
LTNPs	Long-term nonprogressors
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein

NK cells	Natural killer cells
pDCs	Plasmacytoid dendritic cells
Pol	Polymerase
PPARs	Peroxisome proliferator-activated receptors
RT	Reverse Transcriptase
SIV	Simian immunodeficiency virus
T_{CM}	Central memory T cells
T_{fh}	T follicular helper cell
Th	T helper cell
TNF	Tumor necrosis factor
T_{RM}	Tissue resident memory T cells
T_{TM}	Transitional memory T cells

1 INTRODUCTION

Ever since the discovery of the human immunodeficiency virus type 1 (HIV), tremendous efforts have been made in order to diminish the epidemic that has caused the death of millions of people and is presently still affecting over 38 million individuals (1). Antiretroviral treatment (ART) effectively suppresses viral replication and increases peripheral CD4+ T cell counts, the main target cells, resulting in dramatic decrease of disease progression and AIDS-related morbidity (2,3). However, ART is not curative and better strategies for cure are required. Given that a preventive vaccine or complete virus eradication (sterilizing cure) is most unlikely in a near future, focus has instead shifted towards a functional cure; a state of durable control of viral replication and remission from HIV symptoms in the absence of ART without achieving complete eradication of the virus. A very small fraction of HIV-1 infected individuals are able to control viral replication and maintain stable CD4 counts for several years without ART (4-6). These Elite Controllers (ECs) function as a natural model for functional cure and the importance of study the underlying factors of control in these subjects is essential.

This thesis aims to investigate underlying factors associated with spontaneous HIV control, mainly immunological factors and parameters that could contribute to the understanding of how natural control can be achieved. We have studied immunological aspects of viral control in the unique EC cohort. The studies have included investigating the impact of the human gut microbiota in HIV infection and correlating immune activation, antibody profile against HIV, a comprehensive analysis of host protein profile and T cell exhaustion. This broad approach has given us knowledge about parameters involved in spontaneous viral control in ECs.

2 BACKGROUND

2.1 THE HUMAN IMMUNODEFICIENCY VIRUS

2.1.1 Discovery and origin of HIV

The early 1980s mark the period of when the world became aware of HIV as the causative agent of acquired immune deficiency syndrome (AIDS) (7). AIDS was characterized by severe immunosuppression and opportunistic infections such as pneumonia and Kaposi's sarcoma, and more evident in the men who have sex with men community (8). Although the epidemic had already started to gain some global attention some years prior to that, it was not until researchers declared that a retrovirus was causing pathological syndromes including AIDS that the epidemic got world attention. The discovery of HIV, or at the time of the finding named human T-cell leukemia virus (HTLV), granted researchers the Nobel Prize in Physiology or Medicine in 2008 (9).

The majority of HIV infections are caused by HIV type 1 (HIV-1). The less virulent HIV type 2 (HIV-2), which is more prevalent in West Africa, has a poor transmission capacity and infection rate (10,11). Both HIV-1 and HIV-2 have their origin from the simian variant of HIV, termed simian immunodeficiency virus (SIV), that primarily infects non-human primates that function as a natural host to SIV (12). Extensive phylogenetic studies have demonstrated that SIV very likely has been present in non-human primates, such as chimpanzees, for over 32,000 years and have been transmitted to humans through several, independent introductions (13-15). Interestingly, SIV does not typically cause pathogenic events despite high levels of virus replication in their natural host, sooty mangabeys. However, when present in a non-natural host, such as rhesus macaques, SIV usually causes immunodeficiency and increased immune activation, events that are similar to AIDS in HIV-1 infection (16,17). In this thesis, the usage of the term "HIV" refers to HIV-1, unless otherwise specified.

2.1.2 Viral structure and life cycle

HIV is an approx. 100 nm large lentivirus belonging to the retrovirus family. The word "*lente*" is Latin for "slow" and implicates the slow disease progression and long incubation time upon infection with the virus. Common for HIV and all retroviruses is the cone-shaped capsid comprising of the viral protein p24. The capsid contains two copies of positive-sense single-stranded RNA genome (about 9 kilobase pair long) which encodes three polyproteins; Gag, Pol and Env. The Gag proteins (matrix, capsid, nucleocapsid and p6) and Env proteins (surface molecules gp120 and gp41, located on the outer surface on the virus) are structural components that build up the virus. The Pol proteins reverse transcriptase (RT, present in all retroviruses), integrase and protease are enzymes vital for the viral replication cycle. Of particular importance is the RT, it transcribes the viral RNA genome into a DNA strand. In addition, accessory proteins are found which contribute to the assembling of a mature virus particle and are important for immune evasion (Vif, Vpr, Vpu and Nef), as well as Tat and Rev that provide gene regulatory functions (18).

HIV enters the target cell, mainly CD4+ T cells, via conformational binding of Env proteins to cell surface receptor CD4 and co-receptors (CCR5 and CXCR4). Blockage of the co-receptors has been extensively studied as therapeutic interventions. Also, some individuals have a natural 32-base-pair deletion within the coding region of the CCR5 gene (CCR5Δ32), resulting in a non-functional receptor that does not support fusion of virus to the cell (19). The basic aspects of the HIV replication cycle are shown in figure 1. HIV harbors a huge capacity of replication dynamics and possesses a high mutation rate as RT is prone to making numerous errors per genome during just one replication cycle, in addition to error-making during the transcription of DNA to RNA (20,21). The rapid evolution of HIV, in combination with the large number of virions being produced, makes it a difficult target for an effective vaccine and provides an effective way to evade drug therapy and immune surveillance.

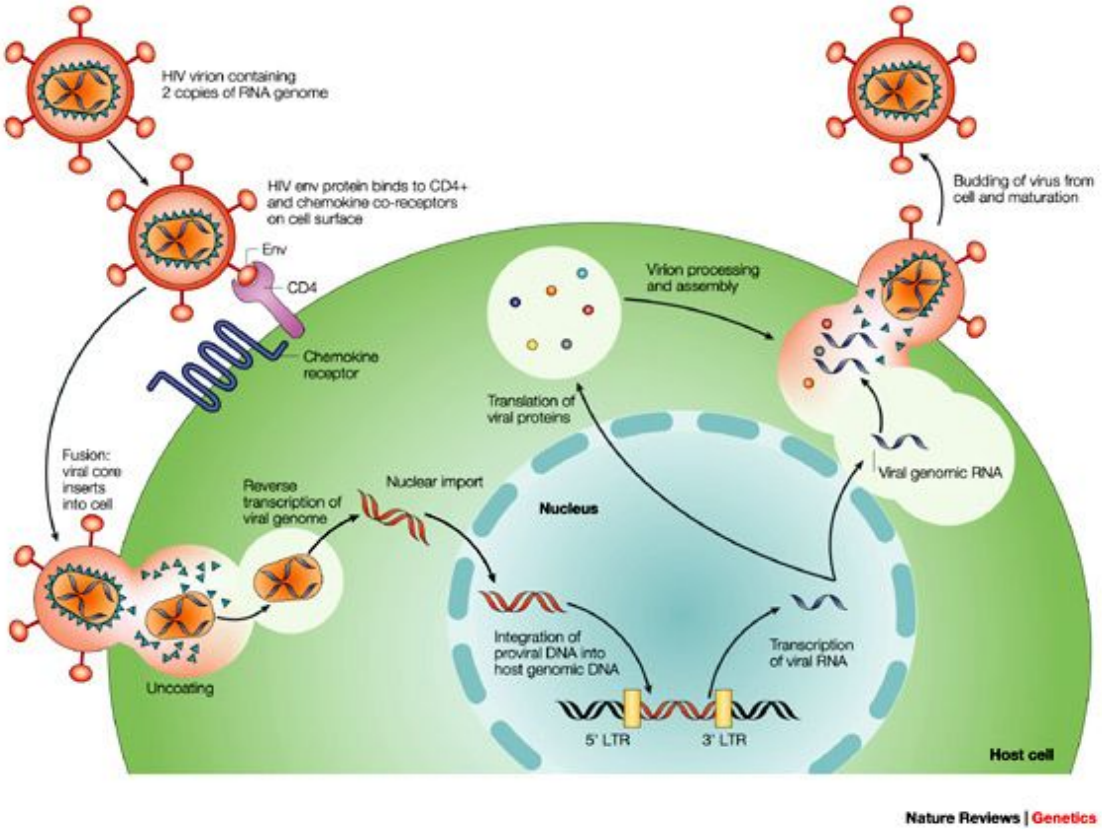

Fig 1. Key aspects of the HIV life cycle. Reprinted with permission (22).

2.1.3 Transmission and course of infection

HIV is a blood-borne infectious agent that targets cells of the immune system. HIV enters the host via body fluids as both free infectious particles and infected cells, and mainly through blood, genital fluids and breast milk. The routes of transmission include sexual contact (hetero- and homosexual intercourse), injection through drug use (e.g. the usage of shared needles), maternal transmission (mother-to-child during pregnancy, delivery and breast feeding) and medical interventions where blood products are transfused. Although the HIV epidemic initially was labeled as a "disease among homosexuals" and more prevalent within the gay

communities, reports from UNAIDS declare that most new infections occur among young women, through heterosexual transmission (1,23).

Following infection, the clinical course of disease progression differs substantially among individuals. Briefly, the infection can be divided into two phases; the acute phase which is then followed by the chronic, clinically "latent" phase. In cases where ART is not available or ineffective, a third phase is included during which the infected individual develops AIDS. The acute phase occurs one-three weeks after initial exposure to the virus and is in a subproportion hallmarked by symptoms, frequently similar to influenza. It is during the acute phase as the virus enters lymph nodes and starts replicating vigorously, establishing itself in vital cells within lymphoid organs. A strong HIV-specific immune response is initialized, resulting in lowering the viremia substantially and entering the chronic phase of the infection. The chronic phase, which can last a few months up to several years, is generally clinically asymptomatic. However, HIV still continues to replicate in CD4+ target cells inside lymph nodes and the beginning of a vicious cycle where the immune system kills virus in the blood, replenishes infected CD4+ T cells and maintains the accompanied immune activation, takes place (24,25). In the events of AIDS, the infected individual has reached a state where the immune system has deteriorated and no longer can maintain the infection. Opportunistic infections arise, where bacterial and other infectious agents seize the chance as the individual no longer has a standing immune defense (26).

2.1.4 HIV persistence

ART has indeed changed HIV infection from being considered a disease with a death sentence to a chronic condition that is considerably manageable. There are several different drugs that target different steps in the viral replication cycle; *entry inhibitors* blocking the binding of virus to surface receptors, *fusion inhibitors* that block the fusion of viral and cellular membrane and *inhibitors* of viral enzymes integrase, protease and RT. Although ART significantly improves the management of HIV infection, ART is not curative. The stage of HIV where it remains hidden from an effective immune response as well as drugs, has been entitled as latent. The term "cellular latency" indicates long-lived, resting, memory CD4+ T cells that harbor HIV provirus integrated into the host's DNA (27-29). The provirus is not actively replicating and immunological inert, making it difficult for ART and the HIV-specific immune response to target those cells. Another difficulty with the resting CD4+ T cells containing viral reservoir is the "sanctuary sites" that shelter these cells. The sites can be located throughout the body in locations such as the central nervous system (CNS), lymph nodes, gut-associated lymphoid tissues (GALT) and genital tract, and is not easily accessible for ART or a HIV-specific immune response (30). While work by Chomont and colleagues identified that central- and transitional memory T cells (T_{CM} and T_{TM}) serve as the major cellular reservoirs for HIV in blood, the latest reservoir research has also focused on sanctuary sites in lymphoid organs (31). Recent work has particularly established that T follicular helper (Tfh) cells in lymph nodes serve as a major component of the HIV reservoirs. Interestingly, work from Louis J. Picker's lab has demonstrated that B cell follicles, the site where Tfh cells are present, are a sanctuary

site for productive and persistent SIV replication. These data suggest that the B cell follicles could function as an immune-privileged barrier where an effective immune response cannot gain access (32).

HIV persistence under ART is indeed a huge obstacle to overcome when aiming for eradication of the virus. Latency is established just days after initial infection and the formation of stable viral reservoirs in resting memory CD4+ T cells might even occur before the individual discovers being infected (33). Some suggestions to target newly-infected cells with ART early during acute infection, in order to prevent establishment of viral reservoirs, is a difficult scenario as many individuals do not seek care until the symptoms arise later. In settings with limited and/or inadequate access to ART, this is even a more difficult issue. The challenges of eliminating HIV reservoirs is in sorts a game of hide-and-seek; one must understand exactly in which sheltered compartments of the body the HIV hides and by what means to eradicate it.

2.2 CONTROL OF HIV INFECTION

Tremendous efforts have been made the past 30 years to find measures against the HIV epidemic (34), however, progress towards a preventative vaccine has been slow, not to mention a great share of disappointments and limited success. The highly noted STEP HIV vaccine trial (also known as HVTN 502 or Merck V520-023), used an adenovirus-vector-based vaccine to induce a HIV-specific cell-mediated immunity in 3'000 non-infected subjects. However, the trial failed and a higher proportion of vaccinated subjects contracted HIV infection compared to non-infected individuals (35). As already mentioned, one major obstacle in inducing HIV-specific immunity and eradication of the virus, besides from genetic variability of viral proteins to escape immune surveillance, are the HIV latent reservoirs. Several therapeutic approaches towards a sterilizing cure, a complete eradication of persistent virus in the body, has been in the pipe-line and a main approach has been the "shock and kill" strategy to target latent reservoirs (36). The approach involves luring out latent HIV by reactivation of memory CD4+ T cells which will then induce the reactivated, infected cells into apoptosis and immune clearance. Meanwhile, the non-infected cells stay protected with ART. Reactivation of cells has been shown to be effectively mediated by histone deacetylase (HDAC) inhibitors, an epigenetic drug that induces expression of integrated provirus (37). However, a study published by Rasmussen et al demonstrate that although the HDAC inhibitor panobinostat disrupts HIV latency, it does not fully reduce the number of latent infected cells, once again showing the difficulties in eradicating latently infected cells (38).

Given that a preventive vaccine or sterilizing cure is unlikely in a close future, focus has instead shifted towards a functional cure; a state of durable control of viral replication and remission from HIV symptoms in absence of ART, without achieving complete eradication of the virus. One case of functional cure has been achieved in the *Berlin patient*, a HIV-infected male who received a stem-cell transplantation from a CCR5Δ32 donor (39). First believed to have achieved complete elimination of the virus in the Berlin patient, Burbelo et al showed low levels of antibody response against RT, gp41and TAT in serum, indicating a unique stability of HIV proteins despite intervention (40). Another case of functional cure was the

"Mississippi baby", an infant subjected to mother-to child transmission as the mother did not obtain treatment during the pregnancy. ART was initiated only 30 hours after birth and remained so for 18 months, in hope that the viral reservoirs had not been established due to early treatment. However, after almost two years without ART and no observed virus levels, viral rebound occurred in the child (41,42). Data from the Mississippi baby case and other similar ones, clearly demonstrate that early ART may restrict viral reservoirs but not eradicate them (43).

2.2.1 Elite Controllers- a model of functional cure

In 2010, Migueles et al reviewed the clinical features of nonprogressive HIV infection where they described the clinical course of two cases with known HIV seropositive status since several years back. Both had been ART-naïve and maintained stable CD4 counts as well as undetectable virus levels (44). Remarkably, a very small subset of HIV-infected individuals is able to control viral replication below detection limits and maintain stable CD4 cell counts for several years throughout infection in the absent of ART. This spontaneous and sustained control of HIV infection represents a very rare and distinct phenotype among HIV-infected subjects termed HIV controllers or ECs, and function as a natural model for functional cure (4-6). The identification of infected subjects that remained AIDS-free, ART-naïve and with high and stable CD4 count has been present since mid 1990s and was then termed long-term nonprogressors (LTNPs) (45-49). Techniques for CD4 determinations came before HIV-RNA quantification in blood was available. LTNPs have been subjected for studies since the early nineties and has, in Professor Sönnerborg's research group at Karolinska Institutet alone, earlier resulted in two theses (50,51). When viral load testing was introduced in the 1990s, it could initially only measure down to 1000 copies/ml. The sensitivity has gradually improved and today, most routine tests can measure down to 20 copies/ml. Thus, upon the introduction of viral load testing it became apparent that the majority of LTNPs were not able to control viral replication despite high CD4 counts and that this group contained in fact an even smaller subgroup. These "true" LTNPs, or ECs, exhibited the same criteria as LTNPs as well as being able to maintain undetectable plasma viral load (by standard clinical assays) for prolonged periods of time (52,53). However, the definition of ECs has been shown to be somewhat more complicated than expected and there is a discrepancy in terminology and inclusion criteria (44). ECs are extremely rare and the prevalence among the HIV-infected population is poorly defined. Hubert et al demonstrated that only one subject of 330 enrolled HIV patients (0.3%) presented a viral load below 20 copies/ml (54). Another study by Lambotte et al demonstrate that 15 of 1551 patients (<1%) were defined as HIV controllers. Although there is no exact number, most studies demonstrate that ECs constitutes less than 1% of the HIV-infected population (55,56).

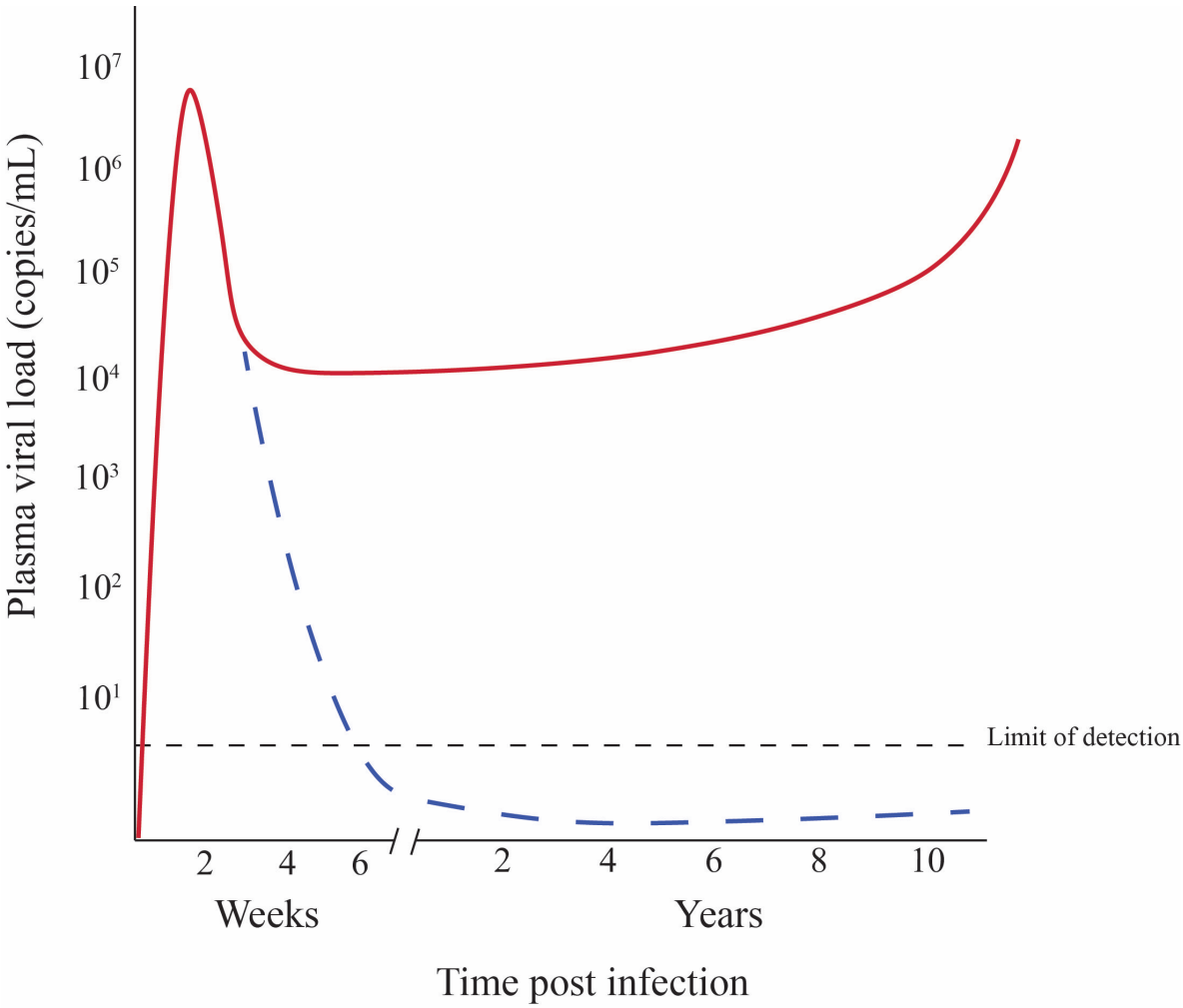

Fig 2. Diagram showing the viral load events of HIV infection. Red line indicates the clinical course of an untreated, chronic disease progression while dotted blue line represent the typical decline in viral load, weeks after infection, observed in ECs. Photo by courtesy of Son Nguyen.

As mentioned, ECs are poorly defined and studies are difficult to compare due to the heterogeneity in study labeling, study design and lengths of follow up (57-59). The basis of inclusion criteria, e.g. number of years as HIV seropositive, opportunistic infections, CD4 counts, HIV RNA levels etc., varies greatly between different EC cohorts (44,60). Even the labeling of ECs or HIV controller differs which contributes to the difficulties of a clear definition. The *HIV controller cohort* (n=19 subjects) from the French National Agency for Research on AIDS (ANRS) is stringent with duration of HIV infection, >10 years, and no opportunistic infections among their controllers while less stringent on levels of plasma viral load for the enrolled subjects (<400 copies/ml). The levels of CD4 counts among the ECs is not encountered (55,61). This is not consistent with for example the *Elite Suppressor cohort* from the Ragon Institute in Boston (n= 66 subjects), that only included subjects that maintain HIV plasma levels below 50 copies/ml and does not encounter CD4 counts nor number of opportunistic infections (62). On the other side of the spectrum is the *LTNP-cohort* from National Institute of Allergy and Infectious Diseases (NIAID), that has identified 63 HIV-infected subjects as controllers with emphasize on stable, non-declining CD4 counts, HIV RNA plasma levels below 50 copies/ml and without ART or any opportunistic infections (63). Some

suggest that one must take into account the virologic status (eg. transient of persistent low level viremia) of ECs in order to decrease the heterogeneity among cohorts (64).

During later years, attempts have been made to standardize the definition. The International HIV Controllers Consortium has established the following criteria for ECs enrollment; HIV-positive for more than one year (determined with standard serological determination), minimum three consecutive HIV RNA determinations below 75 copies/ml during this time period, while ART-naïve and AIDS-free (65). The Concerted Action on SeroConversion on AIDS and Death in Europe collaboration (CASCADE), part of the EuroCoord European network that collects HIV cohort collaboration, suggests that ECs are defined by either of two criteria; 1) HIV positive status for more than one year with minimum three consecutive HIV RNA determinations below 75 copies/ml during this time period AND all previous HIV RNA levels below 1000 copies/ml, or 2) HIV positive status for more than ten years with minimum two consecutive HIV RNA determinations, ≥90% of which are below 400 copies/ml (60). This joint effort to standardize the EC definition could indeed contribute to improve the observed heterogeneity that is so commonly observed among ECs.

2.3 MECHANISMS OF CONTROL

Upon the introduction of viral load testing in the 1990s, when it became apparent that some HIV-infected subjects were able to naturally control the virus infection and maintain stable CD4 counts, it was initially thought that these individuals had been infected with a defective or attenuated virus which contributed to their control status but this was early proven to be exceptions (66,67). Epidemiological factors such as gender, ethnic background and mode of infection appears to have little impact on whether an individual develop EC status (44).

Research has established that the mechanism of control is very likely multifactorial; several different factors in combination contribute to control in different ECs as no infected subjects possess the same mechanism of control, indicating that ECs are a heterogeneous population (65,68). Collectively, the factors of natural control can roughly be divided into viral genetics (viral proteins and enzymes), host genetics and host immune response (innate and adaptive immunity). One cannot emphasize enough the importance of understanding the many different factors of spontaneous control in ECs as such findings can provide critical information for future vaccine and functional cure strategies.

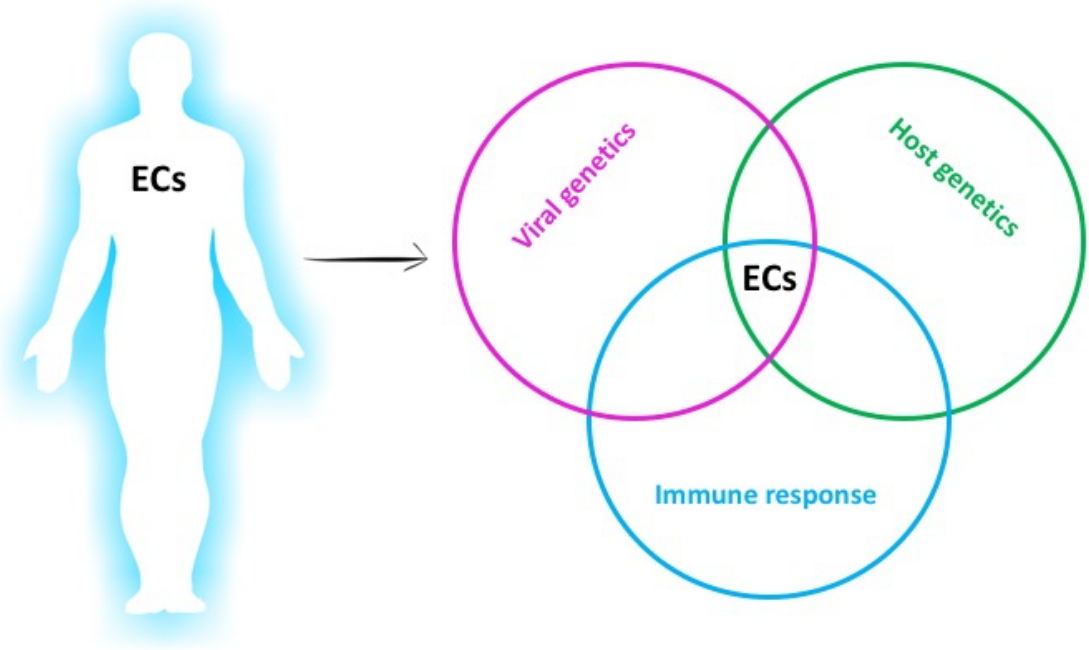

Fig 3. Multifactorial causes of spontaneous HIV control in ECs.

2.3.1 Viral genetics

As mentioned earlier, the initial suggestion for the underlying mechanism of control observed in ECs, was that these individuals had been infected with a defective virus. The non-replication-competent virus was believed to cause the low levels of viremia as the virus was not capable of reaching full viral potential. Indeed, infection with defective or attenuated virus, e.g. mutation and deletion in key functional and accessory HIV genes has been observed in long-term nonprogression or EC (69-72). The most noted case of EC and infection with a defective virus is *The Sidney Blood Bank cohort* which comprises eight subjects who all had been infected with an attenuated, Nef/long terminal repeat-deleted strain virus from one single blood donor. Five of these subjects were subjected to long-term and adequate follow-up and all of them, including the donor, remained asymptomatic for 14-18 years post infection, without any treatment. Noteworthy, one infected subject died five years after infection at age 22, much likely due to causes related to HIV (73,74). Furthermore, studies of whole-genome sequencing of the virus derived from ECs have revealed impairment of viral strains. Functional attenuation of Env, Gag, Pol and Nef genes was described to result in reduced efficiency of cell entry and replication capacity (75-78).

Although data demonstrate the presence of defective HIV in ECs, attenuated virus is present in only a small fraction of ECs, and most subjects harbor fully pathogenic and replication-competent virus (79,80). One study, published by Bailey et al, demonstrated that an infected individual, who developed progressive disease, had been infected by a person who instead had developed EC status (81). Analysis of virus isolate from both individuals revealed replication-competent virus that can cause progressive disease, similar to findings published elsewhere (82). Thus, decreased HIV function may be a hallmark of EC phenotype rather than the cause

of it. Viral factors and reduced fitness of HIV could play a small contributing factor to achieve control but the main contributor is likely host genetic and effective immunity in ECs.

2.3.2 Host genetics

Host genetic factors soon became the subject of research to understand control in ECs. An overrepresentation of certain human leukocyte antigen (HLA) alleles was observed among ECs. HLA genes encodes the major histocompatibility complex (MHC) proteins, expressed on the surface on all cells and is crucial for effective immune response. MHC proteins are responsible for presenting peptides from both intracellular and extracellular pathogens to immune cells and thus eliciting a functional immune response in order to clear the infection. Out of the two classes of MHC, class I is expressed on all cells and present intracellular pathogens, eg. viruses, for recognition by CD8+ T cells and effective clearance by cytotoxic T cells (CTLs). MHC class I is further subdivided into the classical, highly polymorphic HLA-A, HLA-B and HLA-C, and the nonclassical, less polymorphic HLA-E, HLA-F and HLA-G. Genetic variations in HLA alleles has been observed with different disease progression. Large GWAS-studies have independently identified single-nucleotide polymorphism in both HLA-B and HLA-C alleles that is associated with host viral control as well as lack of control (83-85). "Protective" alleles, in particular HLA-B*57 but also HLA-B*27, HLA-B*13 and HLA-B*58:01, are expressed in over 60% of the ECs compared to approximately 40% among infected subjects with rapid disease progression (44,62,86). In contrast, HLA-B*35 is associated with a more rapid disease progression towards AIDS-defining conditions (87,88). HLA-DRB1, belonging to MHC class II, has also been associated with HIV control. In a cohort of HIV controllers (defined as viral load below 2000 copies/ml) and progressors (over 10'000 copies/ml), HLA-DRB1*15:02 was significantly associated with low viremia while HLA-DRB1*03:01 was linked with high viremia (89).

Another noted polymorphism of host genetics has been observed in the CCR5 and CCR2 gene, which encodes co-receptors for cell-mediated entry by HIV. A 32-base pair deletion in the CCR5 gene mediates resistance to infection by R5 tropic virus, and is associated with delayed disease progression and lower viral load (83,90,91). However, the prevalence of CCR5Δ32 among healthy populations appears neither different nor enriched in HIV controller cohorts (55,62,86,92). Noteworthy to mention is that some ECs do not express any of the protective alleles and the opposite, a fraction of ECs that do express protective alleles, still develop progressive disease after a certain time period. Thus, possessing these HLA-alleles is merely not sufficient to maintain EC status and suggests that other factors play a part in achieving sustained viral control.

2.3.3 Innate immunity

The first line of defense against infection, the innate immunity, provide immediate non-adaptive defense that last for a short period of time. Innate immunity is considered "non-specific" due to the rapid and short-lasting nature of responses against any pathogen. The importance of innate immunity in HIV infection, or any infection for that matter, is recruitment

of immune cells via secretion of small molecules called cytokines and chemokines, and activation of the long-lasting and specific adaptive immune system through antigen presentation. Although HIV infection is a chronic disease and thus it appears more relevant to study adaptive immunity, there are unique properties of the innate immune response in ECs that have been described in the context of spontaneous control (figure 4).

Dendritic cells (DCs) are antigen presenting cells, which main function is to process foreign antigens and present it to components of the adaptive immune system, making them a key player in the linking bridge between innate and adaptive immunity. Plasmacytoid DCs (pDCs), a subset of DCs, respond rapidly against viral infections by secreting significant amounts of proinflammatory cytokines such as interferon alpha (IFNα), interleukin-12 (IL-12), tumor necrosis factor (TNFα) and macrophage inflammatory protein (MIP-1β). The secretion of these cytokines promotes inhibition of viral replication and initiate cascades of other innate and adaptive immune responses. Although there is a massive depletion of immune cells during acute HIV infection, that can be partly restored by ART, pDCs are preserved in ECs and maintain a preserved functionality to reduce viral replication (93,94).

Natural killer (NK) cells, a counterpart to the T cells of adaptive immunity, possess anti-HIV properties by producing pro-inflammatory cytokines and cytotoxic molecules. Control of HIV replication is associated with expression of HLA class I alleles in combination with expression of certain killer cell immunoglobulin-like receptors (KIRs, regulatory receptors expressed on surface of NK cells) that serve as ligands to these HLA alleles. Expression of KIR3DS1 together with its ligand HLA-Bw4-80I activates NK cells and results in lower viral load set point and reduced risk of disease progression (95). Similar observation was reported that KIR3DS1$^+$ NK cells are preferentially expressed in HLA-Bw4-80I$^+$ subjects during acute HIV infection and efficiently inhibit viral replication in target cells expressing the HLA-Bw4-80I allele (96). While some HIV controller cohorts observe an enrichment of certain KIRs in their cohort, other cohorts have failed to do so (65,97). In addition, the later study (97) also demonstrated that antiviral capacity of NK cells is not particularly strong nor present in all ECs, indicating that the NK cell-mediated inhibition of viral replication is not a necessity for maintenance of viral control.

2.3.4 Adaptive immunity

The adaptive immune system is a highly specialized system that takes place after the innate immune system and is acquired to respond to pathogens, eliminating them effectively. The adaptive immune system is highly sophisticated to remember and recognize the re-encounter of pathogens, providing a long-term protection. In chronic HIV infection, where there is a constant turnover of virions in different variabilities, the adaptive immune cells (B and T lymphocytes) fails to efficiently clear the infection.

In ECs, an effective HIV-specific immune response is considered to be important for mediating long lasting viral control. Components of the adaptive immune system, neutralizing antibodies and HIV-specific CD4+ and CD8+ T cells, are the dominant factors in the suppression of HIV replication. However, it is still debated upon if one of these components is the cause of an individual developing EC status or if they merely play part in maintaining viral replication. Components of cellular and humoral immunity that mediate viral control are presented in the figure below.

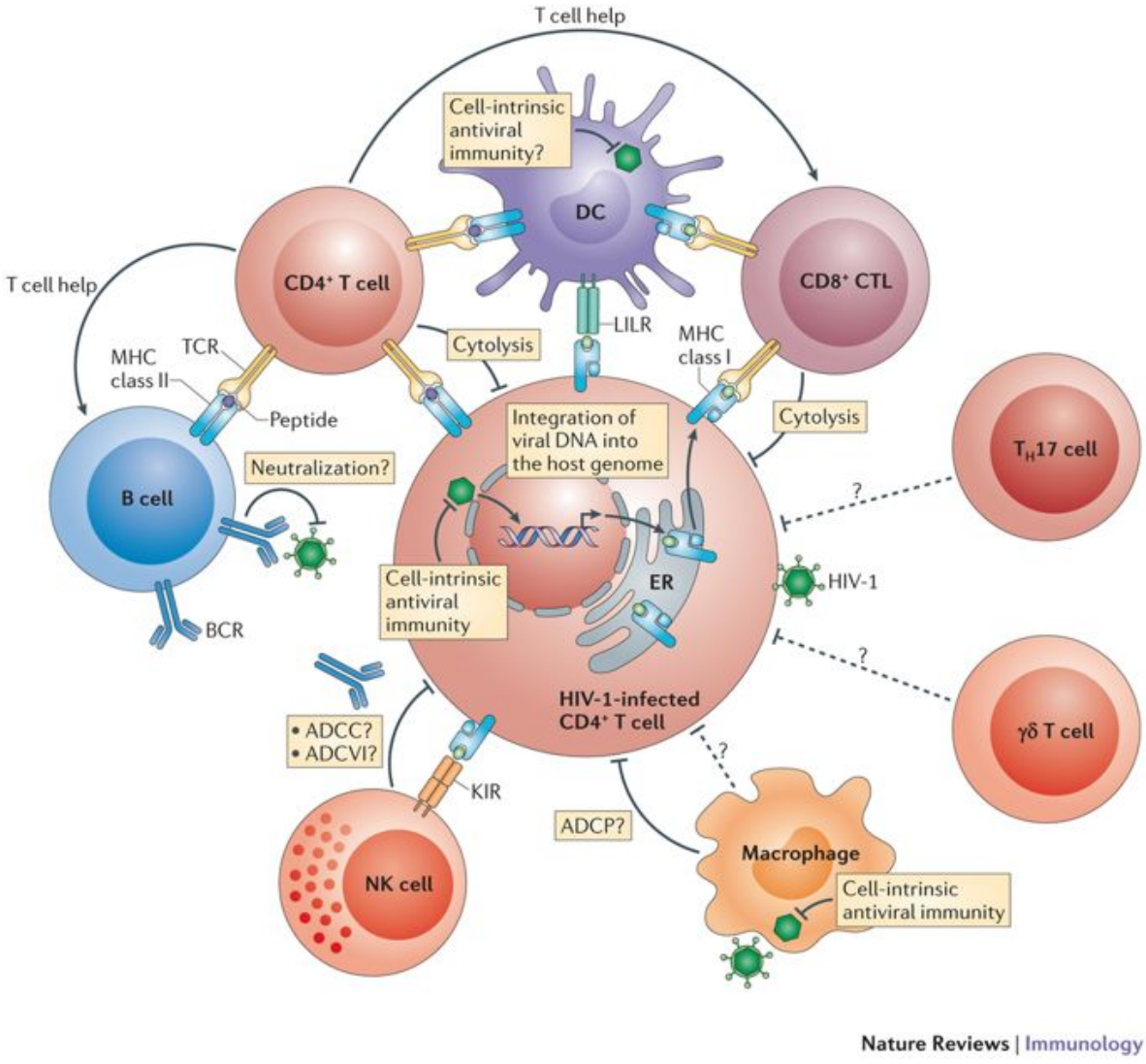

Fig 4. Immune defense mechanisms that contribute to natural HIV control in ECs. Reprinted with permission from (68).

2.3.4.1 Humoral immunity and antibodies

The production of antibodies (Abs) by B lymphocytes constitutes an important part of the adaptive immunity and is responsible for the protection against extracellular pathogens. Besides the progressive depletion of CD4+ T cells, which is partly reconstituted after ART, HIV infection also has an impact on B cells, leading to a dysfunctional and impaired B cell state. The ineffective antibody response against HIV is due to several factors, including high HIV variability, rapid viral mutation rate, poor Ab access to conserved regions of the surface protein Env and functional exhaustion of effector B cells among others (98,99).

Broadly neutralizing Abs (bNAbs) are Abs able to target and neutralize several HIV strains, and differ from conventional Abs that provide specificity against merely one particular viral strain or epitope. bNAbs can target the conserved regions of the Env protein despite the highly variable nature of HIV. In the context of chronic infection (particularly highly mutating viruses, such as HIV and Influenza) and vaccine strategies, bNAbs has been studied extensively (100). Several studies have demonstrated that only a small fraction of ECs are able to produce bNAbs against HIV despite low-grade viral replication. In a cohort of LTNPs, including ECs and viremic controllers, only 7% of the ECs had detectable levels (101-103). Most studies actually conclude that neutralizing Abs are rare in ECs and levels are not different to other HIV-infected subjects with different disease progression rate. Instead, viral evolution has been associated with the development of bNAbs (104), suggesting that levels of bNAbs conjugates with higher viral load (62,105,106). Nevertheless, even though the levels of bNAbs is not to a great extent associated with spontaneous HIV control, understanding the B cell response in ECs and other HIV-infected subjects could provide a blueprint for effective prophylactic vaccine purposes.

Another B cell associated mechanism of clearing infectious agents is the antibody-dependent cell-mediated cytotoxicity (ADCC), a cytolytic mechanism based on Ab-coating an infected cell and thereby promote lysis of target cell by effector cells (mainly NK cells). Viral load was reduced after rectal vaccinal challenge in macaques and associated with ADCC activity, suggesting a protective potential of ADCC in controlling HIV infection (107). In contrast to bNAbs, the measurement of specific Abs that execute ADDC was significantly higher in ECs than in viremic subjects. Specifically, higher levels of Env- and Vpu-specific Abs were observed in ECs than viremics that were capable of activating NK cells and mediating cytolytic activity (105,108). However, evaluating ADCC response has also been demonstrated to be a bit complicated mainly due to the nature of heterogeneity in ECs. The true role of "protective" bNAbs and ADCC Abs in natural HIV control still remains controversial.

2.3.4.2 CD4+ T lymphocytes

CD4+ T lymphocytes, also known as CD4+ T cells or T helper (Th) cells, are a central component of the immune system as its main task is to provide "helper" functions to other components of the immune system. They are crucial for promoting production of different Abs by B cells, and for the activation and proper function of cytolytic CD8+ T cells and macrophages. Upon presentation of foreign antigens from an antigen-presenting cell, the Th cell secretes different cytokines which promote the cell to develop and mature into several different Th lineages (Th17, Th22, Tfh and T regulatory cell among others), depending on the nature of the pathogen and which immune function that requires aid. Briefly described, the CD4+ Th cells are an important factor for regulation of immune responses through coordination of humoral and cellular immunity.

Central for HIV infection is the progressive loss of CD4+ Th cells as it is the primary target of the virus. The size and composition of CD4+ Th cell population decreases both in the circulation and body sites such as the gastrointestinal mucosa. The homeostasis is deeply disturbed and thus the helper function of CD4+ Th cells is also affected (109). In particular, the CD4+ Th cells that recognize HIV-derived peptides (referred to HIV-specific CD4+ T cells) is more affected by the infection than other subsets (110). In the context of control, ECs have a higher functional avidity of HIV-specific CD4+ T cells than infected subjects with higher virus levels in blood. Several studies have demonstrated that HIV-specific CD4+ T cells of ECs secrete IL-2 and IL-21 to a greater extent and are more polyfunctional than in viremic subjects, which is associated with a better capacity of the cytotoxic activities of CD8+ T cells (111,112). A study by Harari et al showed that the frequency of IL-2-producing HIV-specific CD4+ T cells was negatively correlated with levels of viremia, both in blood and lymph node. In individuals with up to 15 months of successful ART, the IL-2 producing cells were significantly lower than in ECs, indicating that HIV-specific CD4+ T cells are not restored despite treatment and could represent a marker of protective immunity (113). This study as well as others, postulates that the observed higher functionality of CD4+ T cells in ECs are a consequence of controlled viral replication rather than the cause of it (114).

The expression of inhibitory immunoregulatory receptors on HIV-specific CD4+ T cells in ECs may also affect the state of natural control. Cytotoxic T lymphocyte antigen 4 (CTLA-4) is expressed on activated T cells and can antagonize the signaling mediated by CD28, thus inhibiting T cell activation and suppressing proper T cell function. CTLA-4 was upregulated on HIV-specific T cells in untreated and treated virally suppressed individuals but not in ECs (115). This indicates that the expression of inhibitory immunoregulatory receptors on HIV-specific CD4+ T cells may suppress a proper proliferation in response to HIV antigen as well as properly function.

2.3.4.3 CD8+ T lymphocytes

A strong and effective HIV-specific T cell immune response, in particular HIV-specific CD8+ T cells, has been demonstrated to be the dominating factor in mediating long lasting viral control. This central component of HIV immune control integrates with and is supported by other immune components (DCs, macrophages, ADCC etc.) to achieve this.

The ability of CD8+ T cell to directly kill infected cells as well as its association with protective HLA class I molecules and viral control, makes CD8+ T cell immunity generally "regarded as the backbone" in mediating the viral regulative activity in ECs (68,116). Studies focus extensively on this type of immune response in order to convert immune protection of HIV to vaccine development. Several research groups have shown that ECs are able to maintain a high frequency of functional HIV-specific CD8+ T cells that are able to effectively kill HIV-infected cells via production of several cytokines and chemokines (117-119). The increased function of these CTLs in ECs is demonstrated by their ability to synthesize large amounts of granulocytic components such as granzyme B and perforin, enabling greater killing capacity of HIV infected cells (63,120). The increased function of CD8+ T cells has been demonstrated to be associated with expression of distinct transcription factors, in particular T-box transcription factor T-bet (T-bet) (121). The enhanced antiviral activity of CD8+ T cells in ECs also appears to be mediated via Gag-restricted epitopes on CD8+ T cells (122). So called CTL escape mutations, i.e. HIV epitopes undergoing mutations in order to escape presentation to CTLs and thereby prevent killing of infected cell, has been demonstrated to be very specific in ECs and often related to protective HLA alleles, which enables CD8+ T cells to control the viral replication (77,123-125). Thus, specific mutated HIV-1 epitopes targeted by CD8+ T cells in combination with certain HLA alleles can be enhancing the polyfunctional effects of viral control.

The persistent antigen exposure to effector T cells during chronic HIV infection leads to an altered differentiation state, called T cell exhaustion, which manifests as loss of effector function and failure to effectively clear the infection (126,127). The sustained up-regulation of multiple inhibitory receptors (PD-1, TIGIT, CTLA-4 among others) maintain this dysfunctional state of T cells and is associated with inefficient control of chronic infections. Extensive studies have shown positive effects of blocking the inhibitory molecules associated with T cell exhaustion; CD8+ T cell immune response has been reversed and reinvigorated, both during chronic viral infections such as HIV and tumor immunity (128). However, the impact of T cell exhaustion on effective CD8+ T cell response (as well as impact on CD4+ T cells) among ECs is poorly understood and further understanding how exhaustion is associated with maintenance of natural HIV control is highly relevant for future HIV eradication strategies.

During recent years, research has also been focused on evaluating the more controversial non-cytolytic, suppressive antiviral activity of CD8+ T cells rather than merely the direct killing capacity. It has been claimed in HIV controllers that a subset of their CD8+ T cells are able to effectively inhibit viral replication in HIV-infected cells ex vivo (129-131). Also, a recent study performed in a simian immunodeficiency virus (SIV) animal model, reported that Chinese

macaque monkeys, upon administration of an oral therapeutic vaccine comprised of inactivated SIV particles and commensal bacteria Lactobacillus plantarum, induced a tolerance against towards SIV (132). This tolerance was observed to be mediated by MHC-E-restricted and non-cytolytic CD8+ T regulatory cells, a concept that is very controversial as there is limited data on CD8+ T regulatory cells as well as MHC class E. However, Carnathan and colleagues were not able to confirm this tolerance when conducting the same experiment in Indian rhesus macaques (133).

Although other research groups have established a suppressive, immunoregulatory CD8+ T cell activity in HIV controllers, there is very limited data on mechanism, transcription factors involved and markers associated with this cell subset. There is some data on CD8+ T regulatory cells in the field of cancer research where a lineage of CD8+ T cells have been shown to inhibit development of autoimmune diseases and regulate anti-tumor immunity (134-136). Although this subset, driven by transcription factor Helios for stable phenotype, is until now only described in mice models regarding mostly tumor immunity (136,137). Personal communication with Dr Hye-Ju Kim revealed a HLA-E-dependent suppressive activity of these cells via involvement of KIRs, similar that has very recently been observed in a small cohort of ECs (138). Several other studies have implicated that immunoregulatory mechanisms might be involved in maintaining low viral replication and preservation of CD4+ T cells. The ECs in a study published by Gaardbo et al displayed elevated percentage of activated T regulatory cells and preserved IL-10 production, suggesting that immunoregulatory cells is playing part in preserving CD4+ T cell levels and the non-progression of HIV infection (139).

As described earlier, HIV is merely a disease of lymphoid tissues and findings from Fukazawa et al have shown that B cell follicles in lymph nodes of rhesus monkey ECs act as sanctuary site for persistent SIV replication (32). As a consequence, potent anti-viral CD8+ T cell responses possess limited ability to enter these sanctuary sites, allowing HIV to sustain in Tfh cells. Buggert et al recently published the first data on lymph node HIV-specific adaptive immunity in ECs. Their data demonstrate that ECs have high levels of HIV-specific CD8+ tissue resident memory T cells (T_{RM}), a non-circulating cell type residing primarily in peripheral tissue, in lymph nodes. The HIV-specific T_{RM} possess enriched effector-related immune genes and signatures in comparison to HIV-specific non-T_{RM}. Their data suggests that CD8+ T cell immunity against HIV of ECs is mostly mediated by T_{RM} in lymph nodes (140,141). Personal communication with Michael R. Betts' research group also describes further interesting findings in lymph nodes of ECs; the majority of CD8+ T cells, including HIV-specific ones, in lymph nodes of ECs do not co-express cytolytic molecules perforin and granzyme B. In addition, these cells exhibit less killing capacity compared to their blood counterparts. Despite the reduced cytolytic capacity, lymph nodes CD8+ T cells from HIV ECs are highly polyfunctional, display a distinctive transcriptional profile, and possess strong viral suppression ability. These results suggest that alternative mechanisms could be involved in the control of viral replication in lymph nodes of HIV ECs.

2.3.5 Immune activation, microbial translocation and the gut microbiota

As described, HIV mainly targets and infects CD4+ T cells but has profound effects on the immune system as a whole. The constant viral replication results in a chronic activation of the immune system which is highly correlated to persistent immunological dysfunction. This systemic immune activation is initiated early during the primary infection and is closely related to disease progression and development of AIDS (142). The characteristic high T cell turnover, B and T cell activation and increased levels of pro-inflammatory cytokines is observed even in patients with suppressed viral replication due to successful ART (143). ART has indeed provided a tremendous success in reducing mortality in HIV-infected individuals but the infected subjects rarely achieve same levels of immune activation as age- and sex matched non-infected healthy controls. They are also at a higher risk for non-AIDS related complications (e.g. cardiovascular diseases, metabolic syndromes, non-AIDS cancer, etc.) than non-infected (144-146). The co-expression of surface markers CD38 and HLA-DR on CD4+ and CD8+ T cells was described early on as a predictor for immune activation and disease progression in HIV infection, and is still today the most prominent used markers of immune activation (142,147,148). In addition, soluble markers such as kynurenine/tryptophan ratio of the indoleamine 2,3-dioxygenase-1 (IDO) pathway and CD14, CD163 and IL-6 are used as a correlate of activated monocytes among other cell types (149). One might postulate that ECs, having suppressed levels of plasma viremia, would therefore also have levels of immune activation similar to healthy non-infected subjects. In contrast, several studies have showed that ECs actually have significantly higher levels of activated T cells than both healthy non-infected and ART-suppressed subjects, as well as a higher portion of activated monocytes (150,151). Pereyra et al demonstrated an increased prevalence of atherosclerosis and monocyte activation marker, soluble CD163, in ECs with no previous record of cardiovascular disease, in comparison with healthy and chronically HIV-infected subjects (152). The abnormally high immune activation seen in natural control of ECs provides a profound risk of non-AIDS complications in ECs.

The role of immune activation as a central feature of HIV immune pathogenesis is very complex and multifaceted. One important contributing factor is the presence of gut microbial products in blood. *Microbial translocation* occurs very early during HIV infection as the gut associated lymphatic tissue (GALT) in gut lumen is damaged due to loss of tight junctions, allowing microbial products from gastrointestinal (GI) tract to enter peripheral tissue and blood circulation (figure 5). GALT harbors a majority of subsets of CD4+ T cell (pro-inflammatory IL-17 and IL-22 producing cells) that are crucial for gut homeostasis and protection of the body from gut microbial products. CD4+ T cells are substantially deleted in the GALT throughout all stages of HIV infection and the skewing of IL-17/IL-22 producing CD4+ T cells is a major contributor of immune activation (153-155). In addition, the gut bacterial products (comprised of peptidoglycan, lipoteichoic acid, lipopolysaccharide (LPS) of the outer wall of gram-negative bacteria, flagellin and ribosomal DNA among others) that leak into the circulatory system causes an activation of the immune components (156-159). Microbial translocation is not solely limited to HIV infection but has also been described in conditions such as

inflammatory bowel disease (IBD), celiac disease and hepatitis C infection (160-164). Microbial translocation is very often associated with a dysbiosis of the composition of the gut microbiota. Considering that our gastrointestinal tract inhabits approx. 10^{14} normal flora of microorganisms living in symbiosis (165), a breach of the protective gut lumen during HIV infection as well as shift or dysbiosis in the composition of normal gut flora could have a huge impact on disease progression and HIV persistence. Several treatment strategies, aiming to decrease damage to the epithelium, enhancing mucosal immunity and restoring gut microbiome in order to reduce systemic immune activation, have been postulated.

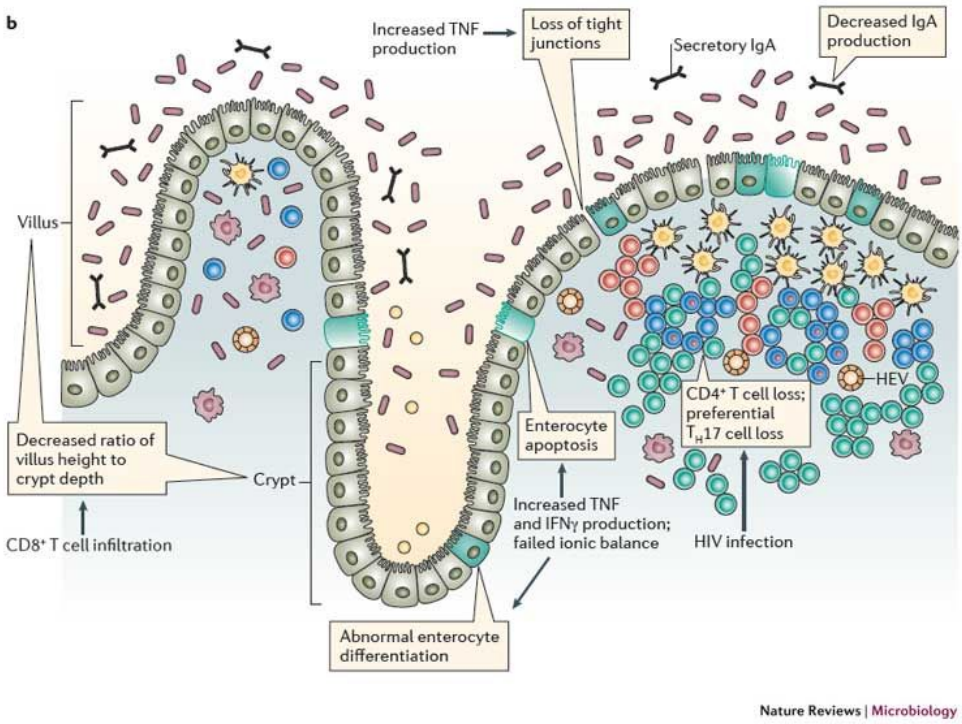

Fig 5. The intestinal epithelium and infiltration of commensal bacteria during HIV. Reprinted with permission from (166).

Most studies relate HIV infection to a decreased bacterial richness and diversity in the gut, and an attributed immune activation driven by microbial translocation(167,168). A seminal paper by Brenchley et al showed that ECs had significant higher levels of markers of microbial translocation than non-infected and yet maintained lower levels than progressors (169). In a small cohort of ECs, IL-17 producing T cells was intact in gut biopsies and displayed only a trend towards monocyte activation (170). A very interesting study by Kim et al investigated the impact of ART on gut immunology in ECs (171). Four ECs were given standardize ART regime for 9 months. Before ART, the number of several CD4+ T cell subsets were similar between ECs, non-infected and long-term ART treated subjects. However, the frequency of Th17 cells was increased compared to non-infected. In addition, ECs displayed higher levels of IL-6 and D-dimer than non-infected, something that ART was not able to reverse.

There are very limited data on the gut microbiota composition in ECs, mostly due to the limited number of ECs and perhaps somewhat difficulty in investigating the gut microbiome as one requires stool sample for the determination. Three papers have described the bacterial composition in spontaneous control of HIV. The first paper, published by Vujkovic-Cvijin et al included only one LTNP so drawing a proper observation and conclusion is difficult (172). The second paper was published from our research group at Karolinska Institutet and included three ECs. It showed that there was a lower interindividual variation in bacterial composition among these three. The ECs differed from viremic subjects regarding bacterial phylum and had a similar composition as non-infected healthy subjects (173). Somewhat controversial data was published by Noguera-Julian et al, where 8 ECs were included in the study, investigating the effect of HIV-infection on the gut microbiome. They found that HIV risk factors, rather than HIV status (including EC status), such as sexual orientation are strongly associated with fecal microbiota composition (174). The most extensive study of the gut microbiome in ECs is probably paper I included in this thesis (175) but the scoop of that paper will be discussed later in accordance. The varied observed data among ECs regarding immune activation, microbial translocation and gut dysbiosis indeed reflects the heterogeneity of this patient population. It further emphases the need for more accurate studies on the role of host-microbiota interaction for this unique patient group.

3 AIMS

The main objective of this thesis was to investigate a variety of different of immune and microbiome parameters associated with spontaneous control of HIV-1 infection in Elite Controllers.

The specific aims were:

Paper I: To investigate if HIV infection affects the gut microbiota in Elite Controllers and patients with progressive HIV infection. Also, to explore the association between composition and functionality of the gut microbiome and systemic inflammation.

Paper II: To determine the antibody profile against HIV-1 proteomes in Elite Controllers, using a modified version of the novel antibody assay "LIPS".

Paper III: To understand the underlying immune mechanisms by which disease progression is prevented in Elite Controllers, by adapting a comprehensive analysis of host transcriptomics- and proteomics data, and clinical phenotypes.

Paper IV: To investigate the expression pattern of inhibitory receptors, associated with T cell exhaustion and defect T cell function, on different CD4+ T cell memory populations in Elite Controllers.

4 MATERIAL AND METHODS

4.1 STUDY DESIGN AND SAMPLE COLLECTION

The premises for all the studies included in this thesis are the study subjects in the *Swedish Elite Controller cohort*. The inclusion criteria for being defined as an EC varies greatly across different cohorts and has created a huge discrepancy among observed data. Therefore, we based our inclusion criteria on recommendations from CASCADE (The Concerted Action on SeroConversion on AIDS and Death in Europe collaboration, part of the EuroCoord European network that collects HIV cohort collaboration), in order to maintain a level of standardization. The Swedish national InfCare database, which includes 100% of the Swedish patients, was used to evaluate all Swedish HIV infected individuals. At the time, out of 22 identified in Sweden as EC, 19 subjects approved to participate in the studies and met either of the two following criteria;

1. HIV-1 positive status for ≥1 year with HIV-1 RNA levels below 75 copies/ml on ≥3 consecutive determinations, spanning over at least 12-month ART-free period with all previous determinations below 1,000 copies/ml (n = 15) or
2. HIV-1 positive status for ≥10 years with ≥2 consecutive HIV-1 RNA level determinations with ≥90% of all HIV-1 RNA determinations below 400 copies/ ml (n = 4)

Four female ECs had been subjected to ART for a very short time due to pregnancy. They were all on treatment for 3.5 months expect for one EC that was on treatment for 14 days. However, the treatment was not within the time-range of the inclusion criteria #1 of what all four EC were subjected to. In addition, n=23 non-infected subjects (age- and sex matched healthy controls), n=19 HIV-1 infected patients on long-term ART (mean 17y on ART) and treatment-naïve, viremic patients (n=38) were recruited. See respective papers for more details about study subjects' demographic and clinical characteristics.

Whole blood was collected from the participants. EDTA-treated blood was further processed with Hypaque-Ficoll density gradient (GE Healthcare) to isolate peripheral blood mononuclear cells (PBMCs). Upon counting, the cells were cryopreserved at-150 ^{o}C in fetal bovine serum containing 10% DMSO (Sigma-Aldrich) at a concentration of 10^7 cells/ml. Serum and plasma were also collected and stored at -80 ^{o}C. In addition, a fecal sample was collected (**Paper I**) in a sterile tube when participant was able to donate sample in connection to their visit at the out-patient clinic and immediately stored at -80 ^{o}C. Participants who were not able to contribute on-site instead provided a fecal sample at a later moment at their homes, using the PSP® Spin Stool DNA sampling tube (Stratec Biomedical). The tube was either delivered to the clinic by the participant or sent by mail, and immediately stored at -80 ^{o}C. 16 ECs and 16 healthy subjects were able to donate a fecal sample. In **Paper I**, treatment-naïve patients from the T-study cohort were included instead of viremic patients from the *Swedish Elite Controller cohort*, mainly due to the availability of fecal samples (n=32).

4.2 METHODOLOGIES

Several different experimental procedures have been used in this thesis in order to approach the many immunological and microbiological aspects of understanding control in HIV ECs. The methods are described in more detail in the respective papers, but below follows a brief summary on the relevant methods and related application in each paper.

4.2.1 Markers of immune activation, microbial translocation and tryptophan catabolism (Paper I)

In **Paper I**, soluble markers of immune activation and microbial translocation was determined in plasma using ELISA; LBP (Hycult Biotech, Netherlands), sCD14 and IL-6 (R&D, Minnesota, USA) and hs-CRP (Abcam, UK). Metabolites of tryptophan catabolism were analyzed in plasma using high-performance liquid chromatography (HPLC) by Bevital, Norway.

4.2.2 Sequencing of gut microbiota and sequence analysis (Paper I)

In order to identify microbial organisms in the gut, we made use of the 16S ribosomal RNA sequencing, a technique based on PCR-amplicons targeting the V3 and V4 region of bacterial 16S rRNA gene. It is a commonly used method for study bacterial phylogeny and taxonomy due to the highly preserved 16S rRNA bacterial gene being present in almost all bacteria. First, DNA was extracted from fecal samples using the PowerSoil DNA Extraction Kit (MO BIO Laboratories, Carlsbad CA, USA) according to the manufacturer's instructions and then sequenced on Illumina™ platform. Second, obtained data was processed using the Mothur, a bioinformatic tool for analyzing 16S rRNA gene sequenses. The bacterial richness estimators (ACE and Chao1) and diversity indices (Shannon and Simpson) were calculated using R/vegan library. In addition, the microbiome function was analyzed with PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States), a bioinformatic tool that provides insight into the metagenomic functional gene content of the microbiome, based on 16S rRNA data and the KEGG database.

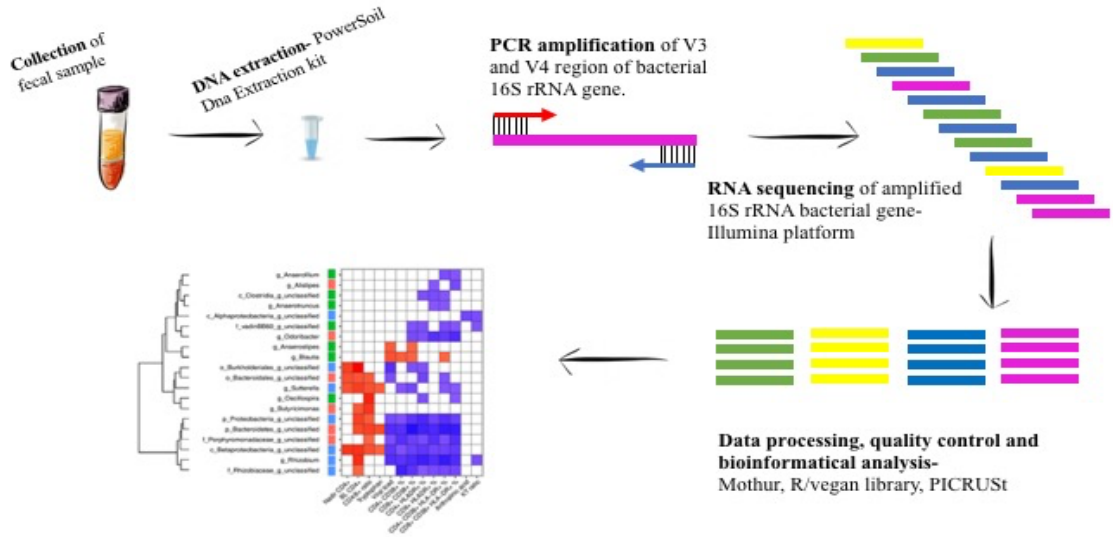

Figure 6. Schematic illustration of a 16S rRNA sequencing and data processing. Heatmap adapted from paper III.

4.2.3 Luciferase immunoprecipitation systems (LIPS) (Paper II)

In **Paper II**, we made use of a modified antibody quantification assay, "luciferase immuno-precipitation systems (LIPS)", to perform antibody profiling against several HIV-1 proteins in ECs. LIPS is a fluid-phase immunoassay with high specificity and sensitivity for conformational epitopes. Briefly, a Renilla luciferase antigen fusion construct against six HIV proteins (p24, reverse transcriptase, integrase, protease, Tat and gp41) was transfected in HEK239 cells to generate a lysate. After mixing the lysate with the subject's plasma and additional reagents, luciferase activity was determined by measuring light-forming units (LU) with Infinite® 200 PRO, Tecan. The antibody response profile was further organized using principal component analysis (PCA) and hierarchical clustering. PCA is a statistical dimension-reduction method that allows reduction of a large set of data to a small set, while still maintaining most of the information. In addition, hierarchical clustering was accompanying PCA to explore hidden patterns in larger data sets and is suitable for large clinical datasets such as described in paper II and III.

4.2.4 RNA sequencing and data processing (Paper III)

In order to identify genes and factors that could play part in HIV control, a comprehensive strategy was addressed involving analysis of host transcriptome, proteomic data and clinical phenotype. The host transcriptome of the study subjects was determined with RNA sequencing, a method widely used to analyze the presence and quantity of the constantly changing RNA molecules in human cells. Briefly described, RNA was isolated from PBMCs or plasma samples using the RNeasy Mini kit (Qiagen, Hilden, Germany) and further quality checked with an Agilent 2100 Bioanalyzer (Agilent Technologies, Germany). The library preparation, e.g. preparation of RNA to make it compatible with the sequencing system, was carried out with poly(A) enrichment in order to isolate messenger RNA fragments (mRNA) and remove ribosomal RNA. The sample was sequenced on HiSeq2500 system (Illumina platform). Data processing, or assembling the transcriptome data, involved several steps and the usage of several bioinformatic tools, figure 7. DE analysis (using Limma R package) and variant calling (GATK software) together with HLA-typing was used to identify differential expressed genes, hence identifying genes that is transcribed to a higher or lower frequency in our samples. The obtained data was further analyzed by gene ontology annotation tool (STRING, UniProt) to identify interactions among the identify differential expressed genes.

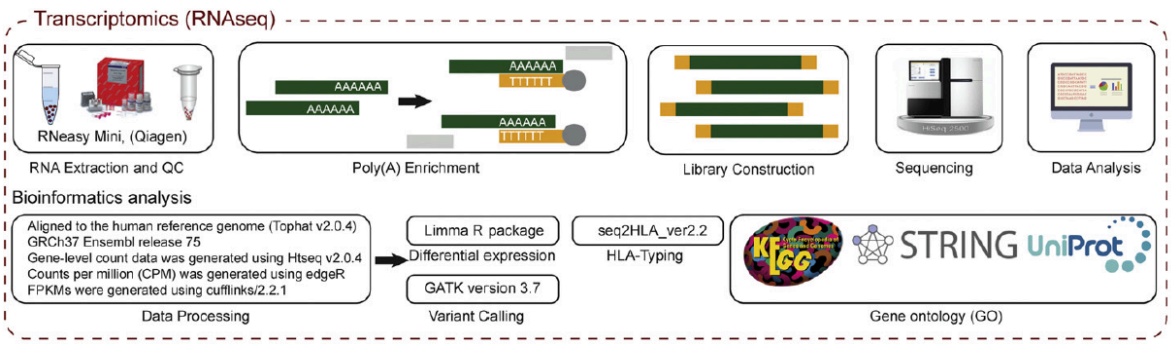

Figure 7. Schematic protocol of RNA sequencing. Adapted from Paper III.

4.2.5 Targeted proteomics (Paper III)

With the obtained data described in section 3.2.4, we performed protein analysis (targeted proteomics) using the proximity extension assay (PEA) (Olink Bioscience, Sweden), an immunoassay for protein quantification and simultaneous detection of several proteins in one sample. Based on the gene ontology annotation of the transcriptome, 92 soluble factors that is part of the Olink Immuno-oncology panel were chosen for further assessment in plasma samples of the study subjects. The panel constituted of proteins involved in cell-surface receptor signaling, programmed cell death and cytokine mediated signaling pathways. The principle of PEA is the binding of an antibody, linked to a proximity probe or oligonucleotide, to your protein of interest. Upon binding, the proximity probes come in close contact and hybridize to each other. By adding a DNA polymerase, an extension of the now hybridizes oligonucleotide and DNA amplicon is created that is quantified by real-time PCR. Proteins of interest was further validated with conventional ELISA (R&D, Minnesota, USA). Data analysis of the proteomics involved generation of protein profile (heatmap) using Qlucore Omics Explorer v3.2.

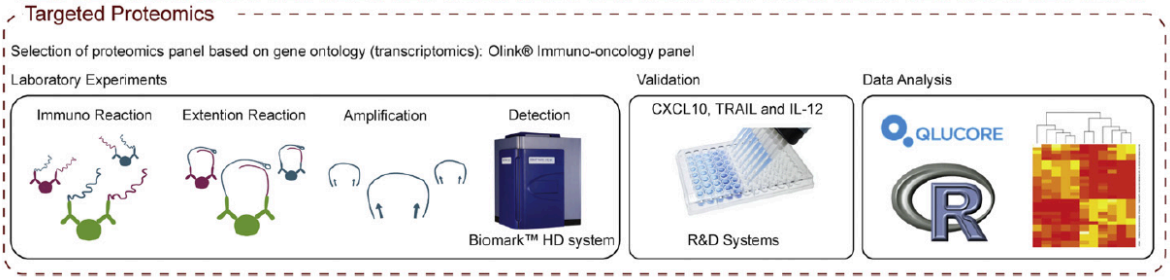

Figure 8. Schematic protocol of Targeted Proteomics. Adapted from paper III.

4.2.6 Flow cytometry (Papers I and IV)

For determination of immune phenotype on lymphocytes, multi-parametric flow cytometry analysis was used. Flow cytometry is a widely used method for simultaneous detection of a single-cell's physical characteristic such as size, granularity and other markers of interest. The working principle of the method is detection of cells labelled with a fluorescent antibody that stream through lasers while flowing in fluid. Flow cytometry allows for detection of several cellular components at single-cell level and can be used for identification of a specific cell population from a complex sample.

As described in **Paper I** and **IV**, study subjects PBMCs were labeled with fluorescent-conjugated monoclonal antibody against cell markers of interest. Upon fixation and permeabilization, cells were acquired on a 4-laser LSR Fortessa (BD Bioscience) and further analyzed with FlowJo software (Treestar) for gating strategy. In **Paper IV**, Simplified Presentation of Incredibly Complex Evaluations (SPICE, developed at NIH, USA) was applied on the flow cytometry data. SPICE is a sophisticated visualization software that allows organization of complex flow cytometry data into clear presentation and evaluation. When investigating several aspects of a large data set, as the co-expression pattern of several receptors on one cell type described in **Paper IV**, SPICE provides both a simple presentation of the data,

as the name implicates, and appropriate statistical testing of the data set (permutation and t-test).

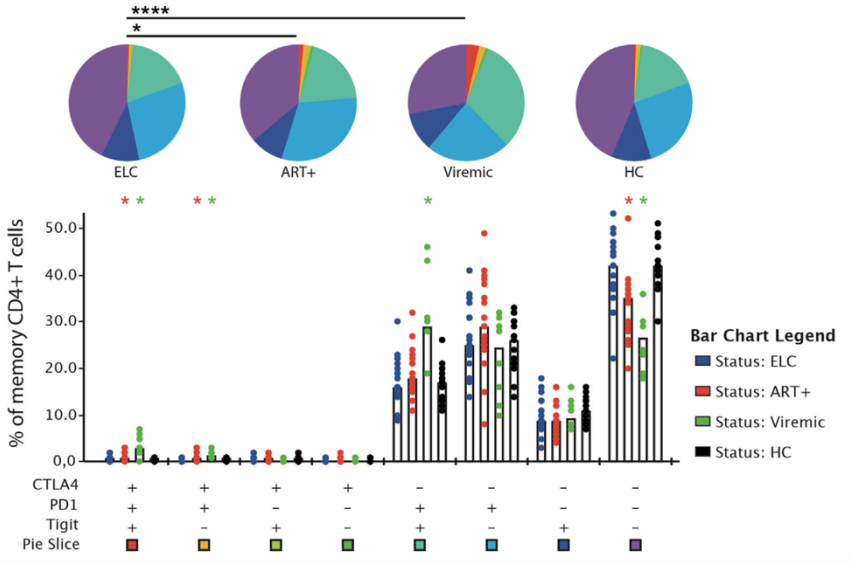

Figure 9. Illustration of a SPICE diagram. Adapted from Paper IV.

4.3 BIOINFORMATIC AND STATISTICAL ANALYSIS

The relevant bioinformatic tools have been described briefly above together with the associated method. Below is a description of the statistics used in respective paper.

Paper I

When comparing inflammation variables, subject demographics, bacterial diversity, richness and composition between two groups, the Mann-Whitney U-test was used while Kruskal-Wallis was applied for multiple group comparison. Spearman's rank test was used for correlation analyses between bacterial genera and composition, functional pathways and markers of inflammation & immune activation. Bacterial genus and function were grouped in heatmap according to Ward hierarchal clustering. Any associations with a Benjamini-Hochberg adjusted p-value cutoff below 0.01 was considered relevant and parameters associated with less than two bacterial species was discarded when plotting heatmap. Lasso regression analysis was used to evaluate the power of classification of bacterial composition between different study groups.

Paper II

For comparison of antibody levels between the different study subject groups, non-parametric Mann-Whitney U-test was used with p-value <0.05 considered significant. PCA and analysis of heatmap (antibody profile) was performed using Qlucore Omics Explorer version 3.2. The PCA was performed with a false discovery rate (FDR) adjusted p (q) <0.05 and the more stringent <0.001 using ANOVA. The statistical analysis was performed with GraphPad Prism software (San Diego CA, USA).

Paper III

When comparing demographic and clinical characteristic data in our study population as well as difference in protein levels, descriptive statistics was used; Mann-Whitney U-test when comparing two groups and Kruskal-Wallis for multiple group comparison (p-value <0.05 considered significant). Hierarchical clustering analysis was performed with a FDR adjusted p (q) <0.001 using ANOVA. The FDR adjusted p (q) <0.05 and the more stringent <0.001 using ANOVA was used as cutoff.

Paper IV

Variables between two study groups were analyzed using Wilcoxon matched-pair rank test whereas two-way ANOVA with Bonferroni correction was applied when analyzing three or more groups. Spearman's rank test was used for correlation analyses. The statistical analysis was performed with GraphPad Prism 6.0. The SPICE software allows for permutation tests when analyzing diversity between the study groups.

4.4 ETHICAL CONSIDERATIONS

All study participants were given both written and oral information about the studies. All participants provided written informed consent in accordance with the Declaration of Helsinki. The Regional Ethical Council in Stockholm, Sweden approved all studies; 2009/1485-31, 2013/1944-31/4 and 2014/920-32. No harm or discomfort was brought to the study subjects more than the circumstances of providing a fecal- and blood sample.

5 RESULTS AND DISCUSSION

Since the discovery that a limited fraction of HIV-infected individuals are able to spontaneously control the infection for long period of time without treatment, extensive studies have been made trying to understand how ECs maintain their control status. Although much attention has been focused on the CD8+ T cell response in the setting of elite control, it is clear that not all ECs bear protective HLA-alleles. As such, it seems as natural HIV control is multifaceted and a comprehensive understanding of underlying factors of elite control is therefore required for future advances in this field. In this thesis, I have focused on investigating several aspects of immunological and microbiological parameters that is related to and could potentially describe the EC-status, including chronic inflammation and gut microbiota dysbiosis, humoral (antibody) profile, T cells dysfunction and gene- and protein expression on a larger scale. It is my hope that the findings presented here can contribute to further understanding of natural HIV control and hopefully contribute to future HIV vaccine and eradication efforts.

PAPER I- RICHER GUT MICROBIOTA WITH DISTINCT METABOLIC PROFILE IN HIV INFECTED ELITE CONTROLLERS

Study background

Progressive HIV infection is hallmarked by depletion of CD4+ T cells in GALT, microbial translocation (leakage of bacterial products to the periphery), gut microbiota dysbiosis and a persistent immune activation. Several studies have related HIV infection to a decreased bacterial richness and diversity in the gut. However, there are very limited studies on the gut microbial composition in spontaneously controlled HIV infection and they are not fully extensive. Thus, in this study we aimed to investigate the gut microbiota composition in ECs, as well as explore the potential relationship between gut bacterial composition and functionality and systemic inflammation.

Results and discussion

We found that ECs and non-infected ("healthy") subjects had similar bacterial richness and diversity in their fecal microbiota (assessed by Chao1 and ACE, and Shannon and Simpson index, respectively) (figure 10). ECs had a lower degree of inter-individual variation of bacterial composition while untreated HIV-infected subjects had a larger spread. We observed a compositional difference in bacteria genera and found that ECs had a unique bacterial signature at genus level. Entirely 17 genera were significantly different from ECs and other study groups (untreated HIV- infected and healthy subjects). Genera such as *Succinivibrio*, *Sutterella* and *Oscillospira* were enriched in ECs while *Blautia* and *Anaerostipes* genera were found to be depleted. Several of these 17 significantly different genera had correlation to markers of immune activation (CD38, HLA-DR). For example, *Sutterella*, a commensal bacteria in the GI tract, was inversely correlated to activated CD4+ and CD8+ T cells. The metagenomic functional content of the gut microbiota, which is predicted with the PICRUSt analysis tool, revealed that several metabolic pathways were significantly different between

ECs and the other study groups. For example, the predicted pathway related to carbohydrate metabolism in the gut bacteria was significantly reduced in ECs compared to both untreated HIV- infected and healthy subjects ($p<0.001$ and $p<0.01$, respectively). The peroxisome proliferator-activated receptors (PPAR)- signaling pathway that play an important role in cellular differentiation and metabolism of carbohydrates, lipids and proteins, was significantly reduced in untreated patients compared to ECs ($p<0.01$). Altogether, this very unique bacterial signature and metabolic profile observed in ECs strongly suggests that bacteria genera is associated with natural HIV control.

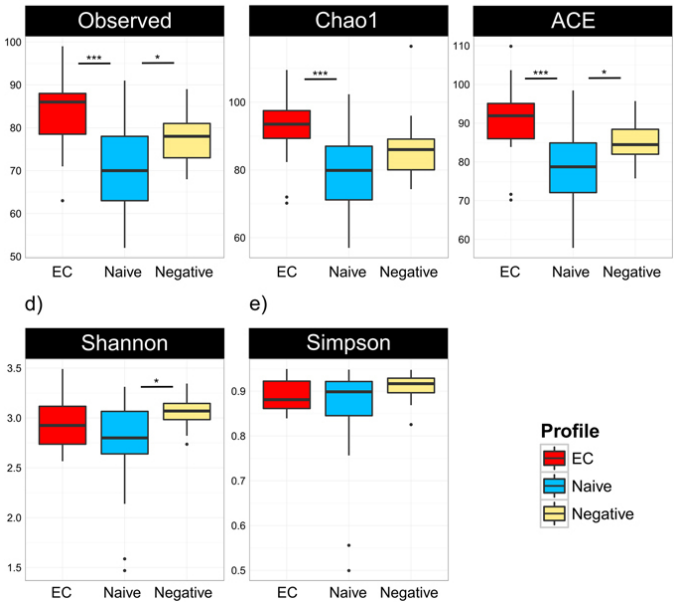

Figure 10. The bacterial richness (assessed by Chao1 and ACE) and diversity (Shannon and Simpson index), in ECs, treatment-naïve HIV-infected and healthy subjects.

PAPER II- QUANTITATIVE HUMORAL PROFILING OF THE HIV-1 PROTEOME IN ELITE CONTROLLERS AND PATIENTS WITH VERY LONG-TERM EFFICIENT ANTIRETROVIRAL THERAPY

Study background

Proper assays for measurement of latent reservoirs is essential for elucidating HIV eradication strategies. Latent infection results in prolonger Ab response and thus measurements of anti-HIV Ab levels, in particular the conformational HIV epitopes, can distinguish HIV-infected individuals harboring different viral reservoirs sizes (40,176). In this project we aimed to investigate the anti-HIV response in ECs by determination of Ab levels, using a modified antibody quantification assay termed "luciferase immuno-precipitation systems" (LIPS).

Results and discussion

We found that the LIPS assay displayed a strong detection of Ab against six HIV antigens (p24, RT, protease, integrase, Tat and gp41). ECs showed significant higher levels of all HIV antigen, except for integrase and Tat, compared to long-term ART suppressed subjects ($p<0.05$) and no statistical difference compared to viremic subjects (figure 11). Analysis of the total Ab response against all six antigens showed similar; ECs has higher median levels ($p=0.001$) than ART-

treated subjects and no difference to viremic subjects. PCA and hierarchal clustering analysis, based on the six HIV antigens, revealed that 68% (13/19 subjects) of ART-treated and 26% (5/19) of ECs clustered together, while most of the ECs (74%, 14/19 subjects) clustered with viremic subjects. Here, we demonstrated that ECs display Ab levels against HIV antigens very similar to viremic subjects and that long-term successful ART is associated with lower levels. We also showed that ECs cluster with subjects with high viremia in regards of total Ab response, indicating that ECs much likely have a constant low-grade viremia that continuously fuel the viral reservoirs and maintain an Ab response. The significant lower Ab levels (against 4 of total 6 HIV antigens) in ART-subjects, compared to ECs, indicate a smaller pool of viral reservoir in those subjects. Indeed, those individuals have been subjected to successful treatment for several years (median 17y, range 13-20) which could explain an effective suppression of viral replication, to a greater extent than ECs may possess by natural means. As ECs maintained lower levels of Ab against Tat and integrase compared to viremics, this might be associated to a lower frequency of integrated HIV DNA in ECs and thus a lower activity of the integrase enzyme (177). The observed heterogeneity in Ab levels among ECs further suggests that this small patient population is indeed very heterogenous, despite our stringent EC inclusion criteria. Determining merely the antibody profile is not sufficient to elucidating factors of viral control and further studies are required.

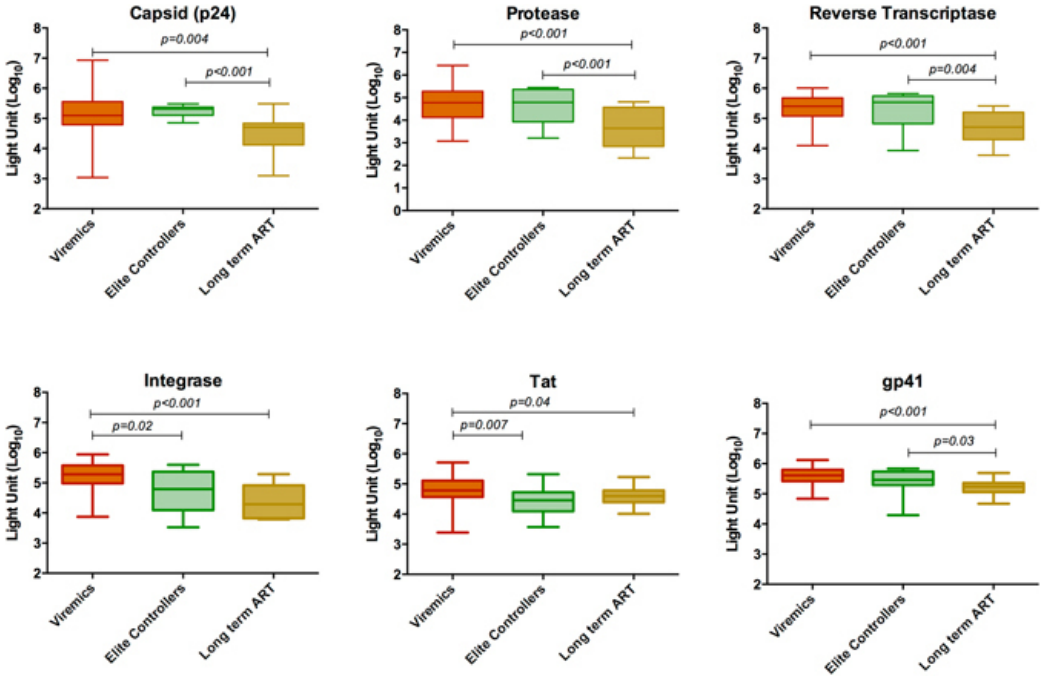

Figure 11. Antibody response against six HIV antigens, in ECs and other HIV- infected individuals.

PAPER III- TRANSCRIPTOMICS AND TARGETED PROTEOMICS ANALYSIS TO GAIN INSIGHTS INTO THE IMMUNE-CONTROL MECHANISMS OF HIV-1 INFECTED ELITE CONTROLLERS

Study background

Following the work in Paper II, we understood the need for a more broad-range analysis in order to investigate factors of HIV control in ECs. Previous studies have mostly focused on a specific, predefined immunological pathway or molecule that could explain the halt of disease progression in ECs. Therefore, we adapted a comprehensive analysis of host transcriptomics, proteomics and clinical phenotypes in ECs, aiming to investigate a systemic, immunological program (rather than a single subset) associated with HIV control.

Results and discussion

HLA typing revealed that 63% of the ECs (12/19) possessed one or more of protective alleles (A*25:01, A*74:01, B*14:02, B*27:05, among others). Some ECs also had HLA alleles related to disease progression (B*35:03, B*53:01 among others). Using Differential Expression (DE) analysis without gender differentiation, 8 protein-coding genes was differentially expressed between ECs and healthy subjects in contrast to 270 protein-coding genes that were differentially expressed between ECs and viremics. DE analysis also revealed downregulation of CXCR6 and SIGLEC, associated with viral entry and formation, in ECs. In addition, with gender-specific DE analysis, we observed 98 transcripts that were differentially expressed between male and female ECs (24 upregulated and 74 down-regulated; 51 transcripts were protein-coding). Among these, osteoclast differentiation, TNF signaling and Toll-like receptor signaling pathway were significantly enriched. No difference was observed between female ECs and female healthy subjects, while 86 transcripts were different between male ECs and healthy males. When investigating protein-coding gene differences in ECs and viremic subjects, we found differences in protein levels involved in cell surface receptor signaling, programmed cell death, cytokine response and cytokine-mediated signaling. In particular CCL4, CCL7 (ligands to CCR5) and members of TNF family was significant different between ECs and viremics (figure 12). In this study, we found that the unique difference in gene expression and transcriptomic profile between ECs and viremic subjects were related to molecular pathways regulating apoptosis, inflammation and cellular differentiation. The elevated level of CCL4, a HIV-suppressive factor produced by CD8+ T cells as well as ligand for CCR5 and thus competitor for the viral binding site, together with the downregulated CXCR6 and SIGLEC genes in ECs, could partly explain the decreased viral susceptibility of T cells in those subjects. Ligands of death receptors (FasL and TRAIL) were lower in EC compared to viremics, implicating a lower susceptibility of Fas-induced cell death (178). The observed gender difference among ECs, that was not related to sex-linked genes, indicate that upon HIV infection, a sex-specific gene process might occur that have an impact in driving immunological mechanisms of viral control. Taken together, these difference between ECs and untreated patients in several molecular pathways suggests they play a synergistic role in HIV control, rather than just one factor making up this control. However, further studies

focusing on functionality in individual molecular pathways, while considering a gender effect, might be beneficial.

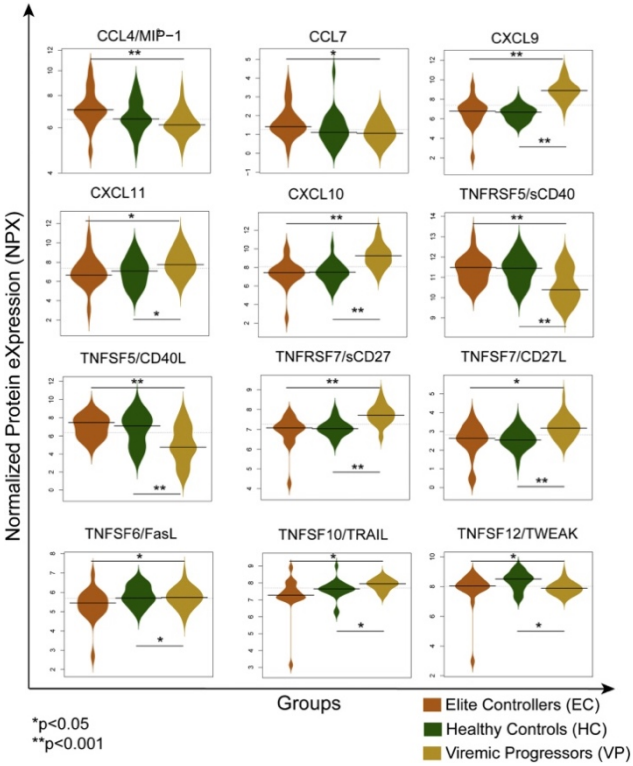

Figure 12. Protein levels involved in cell surface receptor signaling, programmed cell death, cytokine response and cytokine-mediated signaling.

PAPER IV- HUMAN IMMUNODEFICIENCY VIRUS TYPE-1 ELITE CONTROLLERS MAINTAIN LOW CO-EXPRESSION OF INHIBITORY RECEPTORS ON CD4+ T CELLS

Study background

The T cell response play an essential role in controlling HIV infection. However, the persistent HIV-antigen exposure to virus-specific T cells skews them into a dysfunctional state where T cells becomes exhausted (133). CD8+ T cell exhaustion in the context of HIV control has been studies extensively, however, much less is known about exhaustion in CD4+ T cells, the main target cell of HIV. In this study, we aimed to investigate the expression pattern of inhibitory receptors, associated with T cell exhaustion and defective T cell function, on different CD4+ T cell memory populations in ECs.

Results and discussion

Expression of inhibitory molecules associated with T cell exhaustion (PD-1, CTLA-4 and TIGIT) on CD4+ T cells was lower in ECs compare to viremic and long-term treated subjects. CD4+ T cells co-expressing all three inhibitory markers were significantly lower in ECs compared with both long-term treated ($p=0.03$) and viremic ($p=0.002$). The inhibitory receptor expression pattern revealed a significant difference between ECs and viremic and long-term treated but showed no difference to healthy subjects (figure 9). When further dissecting the

CD4+ T cell population, the expression level of PD-1, CTLA-4 and TIGIT showed a similar trend across all subsets with transitional- and effector memory cells displaying the highest expression. There was a correlation between co-expression and T cell activation and clinical parameters; CD38+HLA-DR+ ($r= 0.8$, $p<0.001$), CD4 count ($r= -0.48$, $p<0.001$), viral load ($r=0.37$, $p=0.012$) and CD4/CD8 ratio ($r=-0.37$, $p=0.001$), demonstrating that the frequency of expression is highly associated with immune activation and markers of disease progression. As discussed earlier, some studies have shown that ECs have increased level of immune activation associated with progressive disease and thus one would speculate that the expression levels of T cell exhaustion markers would also be higher in ECs (151,179). Although our data demonstrate a strong association between immune activation and levels of inhibitory markers, only a small fraction of the cells that expressed these markers were activated, implicating that exhausted cells are not activated in ECs. Also, we had previously shown that the CD4/CD8 ratio is a suitable predictor of T cell dysfunction (180,181), which was confirmed in this study. Interestingly, we observed that cells co-expressing PD-1, CTLA-4 and TIGIT was positive associated with CD4+FoxP3+CD25+ cells (markers of conventional regulatory T cells) ($r = 0.58$, $p<0.001$). Despite the strong correlation, only a small fraction of the exhausted cell expressed FoxP3, indicating the presence of another subpopulation of CD4+ T cells that express high levels of inhibitory receptors. In this study, we demonstrated that in natural HIV control, ECs maintained low levels of inhibitory receptors associated with T-cell exhaustion and that ECs are able to maintain low expression levels of marker associated T-cell exhaustion, despite several years of ongoing viral infection. Their ability to sustain a “healthy state” of distinct CD4+ T cell subsets suggest a potential role of these cells in maintaining the control status and further implicates that CD4+ T cell exhaustion is an important component of effective HIV control.

6 CONCLUSION AND FUTURE PERSPECTIVE

The existence of ECs provides evidence that long-lasting control of HIV replication is achievable without ART. ECs are a central natural model for functional cure and has been subject for research for many years, in hope that findings could direct research into novel therapeutics and vaccine strategies. However, understanding durable control has been proven to be more difficult than anticipated. The heterogeneity among these individuals and the multifaceted immunological aspects of control demonstrate that a more comprehensive analysis approach is required. This thesis has defined several immunological and microbiological parameters associated with spontaneous HIV control by focusing on a broad analysis strategy. Here, humoral (antibody) profile, chronic inflammation and gut microbiota dysbiosis, T cell exhaustion, and gene- and protein expression have been investigated in ECs in order to further define the multifactorial aspects related to durable HIV control.

The main conclusion from each paper included in this thesis are:

- ECs have a very different gut microbiota composition than individuals with progressive infection and resembles those that are not HIV infected. Their very unique bacterial signature, metabolic profile and rich bacterial gut microbiota suggests they are important contributors in the facilitation of natural HIV control (**Paper I**).

- The antibody profile of the HIV proteomes (p24, protease, RT, integrase, Tat and gp41) in ECs suggest an ongoing low-grade viral replication, probably related to the size of the HIV reservoirs. The observed difference in antibody levels among ECs demonstrate a heterogeneity in this group (**Paper II**).

- Gene expression analysis and proteomics profiling of ECs suggest a synergetic interplay of cell surface receptor signaling pathway, programmed cell death, response to cytokine and cytokine-mediated signaling in the mediation of spontaneous HIV control (**Paper III**).

- ECs maintain low expression of inhibitory receptors (CTLA-4, PD-1, TIGIT) associated with T cell exhaustion, on their CD4+ T cells. Despite prolonged ongoing viral infection, ECs harbor a “healthy state” of CD4+ T cells that might play part in the maintenance of their control status (**Paper IV**).

Altogether, these conclusions describe the complexity of ECs status and identify factors associated with control that is of importance for further studies. We understand that merely focusing on one single, defined parameter is not sufficient enough and that there is probably an interplay between T cell immunity, Abs, bacteria in the gut and gene- and protein expression. Of course, from these studies, we are not able to conclude what is the hen and what is the egg, i.e. if one of the above-mentioned factors is the dominating one and the other a consequence of that factor, or if there is another unidentified factor that makes up this control. Nevertheless,

we have observed several different immunological aspects in ECs and can conclude that several factors affect each other in mediating durable HIV control.

There are some limitations with the studies presented in this thesis. The most noteworthy to mention is the limited number of included subjects that hold the EC status. As discussed earlier, the prevalence of EC among the HIV-infected population is indeed significant low (less than 1%) and the huge discrepancy of the EC-definition definitely narrows that number. In our cohort, we have adapted a strict definition of ECs and thus limiting the number of subjects that meet this criterion. That said, one vital strength of our cohort, that has enabled us to identify and correctly include subjects, is the usage of the InfCare HIV national database. In Sweden, every HIV-infected subject is enrolled in this database and makes regular visits to the infection clinic where determinations of several parameters, including VL and CD4 count, is standardized. This information has enables us to follow a potential EC for several years in order to determine if he/she truly meets the EC criteria. Thus, the number of ECs in our cohort might be low but they are very well-defined.

I have previously discussed the importance of CD8+ T cell in maintaining the viral control in ECs and although the studies included in this thesis do not address the research question, it is noteworthy to mention that we aimed to define alternative, non-classical, effector functions of CD8+ T cells mediating HIV control. Despite extensive work, we were unsuccessful to provide any such data. During my PhD studies, I have received the question "why not put these ECs on treatment, for a preventative measure?". Although it is not the scope of this thesis, I consider this question important to address. Several factors speak for ECs being a suitable model for functional cure, while some studies suggest the opposite and explain "negative effects" with the EC status (182). ECs are able to control plasma viremia to almost undetectable levels but HIV RNA and integrated HIV DNA have been detected in peripheral blood mononuclear cells as well as occasional viral "blips" with detectable viral levels (183). These data suggest that there is a constant, low-grade viral replication for several years that can have a huge impact on fueling a constant immune activation in ECs. Also, detectable levels have been shown in rectal cells (184), demonstrating that even though ECs maintain low levels of viral replication, the levels are indeed detectable and the virus is replication competent. The observed negative effects of EC status have developed into suggestions to put these individuals on ART. Some studies have demonstrated beneficial effects of ECs receiving ART (185-187); reduced HIV RNA levels than the initial low levels, reduced levels of immune activation and dysfunction in both blood and gut. However, long-term follow up of the treated ECs in the study by Hatano et al demonstrate that the benefits of ART in ECs are considerable low and does not outweigh the negative effects such as adverse side effects of drug, psychological effects, etc. (Dr Marcus Buggert, personal communication with Dr Steven Deeks). By citing Cokerham et al that "ECs and post-treatment-controllers provide additional evidence that functional cure is possible…they give us clues about potential novel interventions that might achieve long-term control of HIV viremia…and suggest that complete eradication of replication-competent HIV from the body may not necessary for long-term remission from the effects of HIV and the suppression of viral replication in the absence of ART" (188). This statement has shifted the

authors initial opinion that ECs are not necessarily the most suitable model for functional cure to actually confirm that ECs do indeed hold important clues on how to achieve sustained long-term viral control without ART. This indeed demonstrates, despite several studies on this unique group of subjects, the importance of the continued study of the characteristic features of ECs and defining the protective factors contributing to sustained control as well as factors associated with disease progression, in the context of translating mechanism of viral control to future cure strategies.

7 ACKNOWLEDGEMENTS

First, I would like to send my heartiest gratitude to the study subjects involved in these studies. Without your invaluable contribution this thesis would not be possible and I am forever grateful. Second, I am also reflecting about what this experience as a PhD student at Karolinska Institutet has brought me. Undergoing PhD studies requires an enormous amount of dedication, commitment and patience, and that is indeed something I have experienced over the years. It has been far from a straight path; projects have failed, time and energy associated with disappointments and personal events have deeply affected my work. A chronic degenerative eye disorder as well as a tough pregnancy and aftermath has had a huge impact on my studies, both physically and emotionally. There have been moments when I was not sure if I would be able to finish my studies but due to the enormous support from my research group, friends and family, it was made possible. For that I am forever grateful to you.

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