Disease progression in Human Immunodeficiency Virus type 1 infected viraemic controllers

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Disease Progression in Human Immunodeficiency Virus type 1 Infected Viraemic Controllers

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ABSTRACT

Background

The mechanism of $CD4^+$ T-cell decline in Human Immunodeficiency Virus-1 (HIV-1) infection is unclear, but the association with plasma HIV-1 RNA-load suggests viral replication is involved. Viraemic controller patients with low HIV-1 RNA-loads (<2000 copies/ml) typically maintain good $CD4^+$ T-cell counts (>450 $CD4^+$ T-cells/mm^3). However, within a cohort of 86 viraemic controllers, a subgroup (18 'discord controllers') was identified with low $CD4^+$ T-cell counts (<450 $CD4^+$ T-cells/mm^3) which present clinical uncertainty. The underlying mechanism accounting for $CD4^+$ T-cell decline in the face of low or undetectable HIV-1 RNA-loads is unknown. The objective of the work described in this thesis was to investigate the virological and host immune system dynamics in discord controllers compared with typical controllers.

Method

Epidemiological features, HIV-1 subtype, cellular HIV-1 DNA-load, T-cell populations ($CD4^+$/$CD8^+$ naïve/ central-memory/ effector-memory subsets; CD45RA/RO ± CD62L) and T-cell activation markers (CD38, HLA-DR) were examined for discord controllers and typical controllers as well as progressors with HIV-1 RNA-load >10000 copies/ml, <450 $CD4^+$ T-cells/mm^3.

Results

Discord controllers and typical controllers were similar, based on epidemiological features and viral subtype distribution. They resembled progressors, showing high HIV-1 DNA-load, depletion of naïve $CD4^+$ T-cells and higher activation in all $CD4^+$ T-cell subsets. However, the $CD8^+$ T-cell compartment in discord controllers was similar to typical controllers with preserved naïve $CD8^+$ T-cells and low level $CD8^+$ T-cells activation.

Conclusion

The data presented in this thesis is consistent with a relationship between $CD4^+$ T-cell activation, HIV-1 DNA-load and disease progression but not HIV-1 RNA-load. This suggests that in viraemic controllers, HIV-1 DNA-load may be a better marker of viral replication and disease progression than HIV-1 RNA-load. Furthermore, low level $CD8^+$ T-cell activation correlate with low plasma HIV-1 RNA-load but not with HIV-1 DNA-load.

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ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome	LTR	Long terminal repeat
ART	Antiretroviral treatment	mRNA	Messenger ribonucleoic acid
CCR	C-C chemokine receptor	MSM	Men who have sex with men
CRF	Circulating recombinant form	nAb	Neutralising antibody
CXCR	C-X-C chemokine receptor	Nef	Negative regulatory factor
CTL	Cytotoxic T lymphocyte	p	Protein
DNA	Deoxyribonucleic acid	PCR	Polymerase chain reaction
dsDNA	Double stranded DNA	PBMC	Peripheral blood mononuclear cell
Env	Envelope glycoprotein	PIC	Preintegration complex
env	Gene encoding Env	*Pol*	Polymerase gene
Gag	Group specific antigen gene	qPCR	Quantitative polymerase chain
gp	Glycoprotein	Rev	Regulator of virion expression
HAART	Highly active antiretroviral treatment	RNA	Ribonucleoic acid
HIV-1	Human immunodeficiency virus Type 1	RT	Reverse transcriptase
HLA	Human leukocyte antigen	SIV	Simian immunodeficiency virus
HSV	Herpes simplex virus	STI	Sexually transmitted infection
IFN	Interferon	Tat	Trans-activator of transcription
IL	Interleukin	TCR	T-cell receptor
IQR	Interquartile range	UNAIDS	Joint United Nations Programme on HIV/AIDS
IVDU	Intravenous drug user	URF	Unique recombinant form
LPS	Lipo-polysaccharide	Vpr	Viral protein R
LTNP	Long-term nonprogressor	Vpu	Viral protein U

CHAPTER 1

General Introduction

Human Immunodeficiency Virus (HIV) infection, the cause of AIDS (Acquired Immune Deficiency Syndrome), continues to be one of the most significant infectious diseases globally. An estimated 34 million people worldwide were thought to have been living with HIV infection at the end of 2010 [1]. The advent of antiretroviral treatment (ART) has reduced the rate of AIDS related deaths [2, 3]. However, access to therapy is not universal and despite huge strides having been made in understanding the basic biology of the virus, progress is slow in terms of finding curative treatments or an effective vaccine [4]. Therefore, it is clear that HIV infection will continue to be a significant public health problem for many years to come.

Ever since HIV-1 was first discovered, the reasons for its epidemic spread and severe pathogenicity have been studied intensively. Here is a summary of what is currently known about the virus with a focus on pathogenesis, natural history and clinical monitoring of disease. In addition, a group of patients termed 'viraemic controllers' are described who are infected with the virus, but in whom only low levels of the virus are found in blood.

1.1 Origin and Classification of HIV-1

In 1983, when Montagnier and colleagues at the Pasteur Institute first isolated the virus responsible for the AIDS epidemic, one of the first features noted about the virus was its reverse transcriptase (RT) activity [5]. This property placed the new agent in the family *Retroviridae*. The subsequent cloning and sequencing, revealing the genetic organisation of the virus, further placed it in the lentivirus genus, with close homology to non-human primate lentiviruses [6]. As the name suggests (*lenti* - Latin for slow), lentivirus infection gives rise to a chronic course of disease with a long asymptomatic period.

Two types of HIV have since been described: type 1 (HIV-1) and type 2 (HIV-2). HIV-2 is less virulent, has lower transmission rates and has remained geographically limited to West Africa, with the highest prevalence rates in Guinea-Bissau and Senegal [7]. HIV-1 is sub divided into three distinct lineages: groups M (major), O (outlier) and N (non-M, non-O) [8]. It has been observed that various species of wild primates in Africa frequently harbour distinct lentiviruses (Simian Immunodeficiency Viruses, SIV) including African green monkeys, sooty mangabeys, mandrills, sykes, chimpanzees and gorillas [9]. SIV infection of these African nonhuman primates is often referred to as 'natural infection' as it is thought to represent an ancient virus that has evolved with its primate host and does not typically give rise to disease [10]. Genetic analysis of HIV with SIV led to the current belief that HIV-1 and HIV-2 represent at least three separate transfers of SIV from nonhuman primates [11]. A phylogenetic tree of HIV and various SIV is shown in Figure 1.1, together with a description of these transfers. More

recently a fourth subgroup of HIV-1, called group P, was detected in two patients originally from the Cameroon [12, 13]. Like HIV-1 group O, it is thought to originate from an independent transmission of SIV from gorillas [12].

Group M HIV-1 is further subdivided into subtypes or clades lettered A-D, F-H, J and K on the basis of phylogenetic analysis of the gene encoding the envelope glycoproteins (*env*). The gene and protein sequences within a subtype are more closely related to one another than to the genes and proteins from other subtypes. Subtypes A and F are divided into sub-subtypes A1-A4, and F1 and F2, respectively, since each set of sub-subtypes is more closely related to each other than to other subtypes [14, 15]. Subtypes B and D are also more closely related to each other than to other subtypes, but continue to be named as such to retain consistency with earlier published work [14]. This categorisation of HIV-1 into genetic subtypes based on viral sequencing is a powerful epidemiological tool with which to evaluate the HIV-1 epidemic.

This thesis, including the rest of Chapter 1, will focus on HIV-1 and will only refer to 'HIV' generally if the paper being cited has not specified which type.

Infection with differing HIV-1 isolates in an individual has been documented in small populations of high-risk individuals [16, 17]. All such cases were once thought to be the result of exposure to different isolates more or less simultaneously, before the immune response had matured. However, it is now thought that 'superinfection' also occurs as a result of infection with the second isolate some time after infection with the initial isolate. This highlights the insufficiency of the immune response to the first isolate in protecting against superinfection [17, 18].

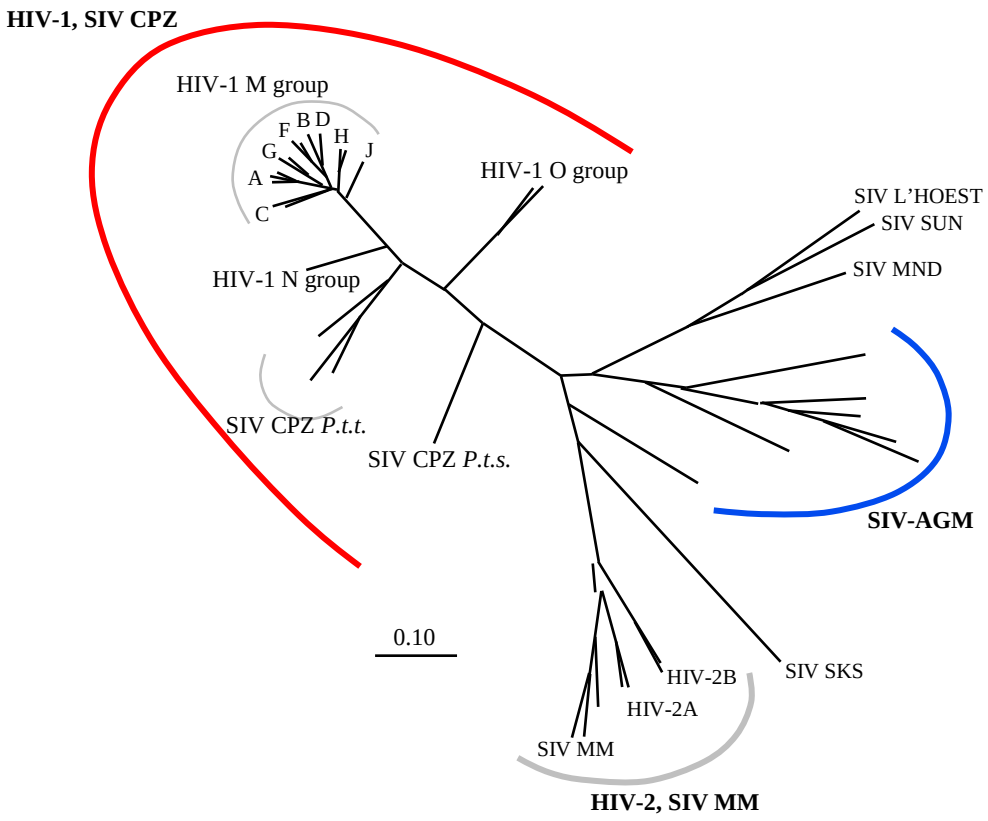


Figure 1.1 Phylogenetic relationship of primate lentiviruses. Figure taken from Kuiken *et al.* [19]. Alignment of *pol* gene sequences of HIV-1, HIV-2 and SIV isolates; *pol* sequences were used as they are relatively conserved amongst lentiviruses even when other regions of the genome differ widely. Within HIV-1, the subtypes in group M are phylogenetically equidistant from one another. HIV-1 group M and N are phylogenetically similar to SIV which infects central chimpanzees, *Pan troglodytes troglodytes* (SIV CPZ *P.t.t.)* [20]. HIV-1 group O, which is phylogenetically closely related but distinct from these groups, has been found to be related to Gorilla SIV [21]. Each of these appear distinct from SIV CPZ *Pan troglodytes schweinfurthii* (SIV CPZ *P.t.s.).* HIV-2 is related to SIV which infects sooty mangabeys, *Cercocebus atys atys* (SIV MM) [22]. These isolates are phylogenetically distinct from other SIV isolates including those which infect the African green monkey (SIV AGM), sykes (SIV SKS) and mandrills (SIV MND).

In addition to the HIV-1 subtypes described, there are many inter-subtype recombinant isolates, often restricted to the dually infected individual in which they arose. These forms contain clearly delineated sections from different subtypes and are thought to result from genetic recombination of viruses of differing subtypes (see Section 1.4). If the recombinant virus is fully sequenced and found in three or more epidemiologically unlinked individuals it can be classified as a circulating recombinant form (CRF); isolates are referred to as unique recombinant forms (URFs) if these criteria are not met. The CRFs are labelled with numbers instead of letters, in the order in which they were first described, followed by letters indicating the subtypes involved. They must share an identical mosaic structure, i.e. they descend from the same recombination events [8]. For example, CRF01_AE was the first fully described CRF and found to contain components of subtype A and E, although no 'pure' full-length genome has been found for subtype E [8]. There are now more than 50 CRFs worldwide [23]. If three or more subtypes are found to make up a recombinant form, the isolate is designated as 'cpx' rather than a list of subtypes involved.

1.1.1 HIV-1 Genomic Diversity

The HIV-1 genome is extraordinarily diverse. Genetic variation within a subtype is around 8-17%, whereas variation between subtypes is usually 17-35% depending on which region of the genome is examined [24]. The greatest variability occurs in the region of the genome which encodes the envelope glycoprotein 120 (gp120) (see Section 1.3) [25]. Even within a single infected individual huge heterogeneity is observed, with this range of isolates being referred to as the 'quasispecies'. Incredibly, the genetic diversity of the HIV-1 quasispecies in an infected individual at the late stages of disease is comparable to the global annual genetic variation of the influenza virus [25]. This is particularly surprising since most infections are thought to arise from a single 'founder' virus [26, 27]. This genetic heterogeneity results from the rapid replication kinetics of HIV-1, mutations during viral replication, viral genomic recombination (see Section 1.4) and immune selection pressures (see Section 1.7) [28, 29]. Such diversity allows for selection of variants which readily escape control by host immune responses and ART, and accounts for much of the difficulty in vaccine development and in developing therapies against HIV-1.

There is some evidence that genetic variation between subtypes or CRFs may result in differing viral properties, such as cellular tropism, kinetics of viral replication or differences in viral pathogenesis. For example, Kanki *et al.* found that female sex workers in Senegal infected with a non-A subtype were eight times more likely to develop AIDS than were those infected with subtype A over the study period, suggesting possible decreased pathogenicity of subtype A

[30]. An increased pathogenicity of subtype D isolates has been reported by several groups [31-33]. Infection with subtype CRF01_AE virus, commonly seen in Thailand, has been reported to cause more rapid progression and shorter median survival than that which is described in ART-naïve individuals in high income countries where other subtypes predominate [34, 35]. The differing immunological status of the population in Thailand may account for these differences rather than viral subtype [35]. It has also been observed that certain subtypes or CRFs are associated with specific modes of transmission [36]; these observations will be discussed in the next Section.

1.2 HIV-1 Epidemiology

1.2.1 HIV-1 Risk Factors and Transmission

The AIDS epidemic was first identified in the United States within the men having sex with men (MSM) risk group, in men and boys with haemophilia exposed to contaminated clotting factors, in recipients of blood transfusions and in intravenous drug users (IVDU) [37]. Transmission as a result of exposure to contaminated blood products has virtually disappeared with the introduction of screening assays. For example, current blood transfusion screening assays mean the risk of transfusion-transmitted HIV is estimated to be 1 case per 1.5 million transfusions [38]. However, the epidemic has since grown at an alarming rate with the major risk factors being heterosexual and homosexual intercourse, vertical transmission and intravenous drug use. Heterosexual intercourse is the primary mode of transmission in sub-Saharan Africa, although infections in MSM and IVDU are starting to be seen [39]. In Asia, the epidemic had long been concentrated in high risk populations, including IVDU, sex workers and their clients and MSM. However, transmission is now increasingly seen in lower risk populations, with heterosexual transmission now being the predominant mode of spread in Asia [40]. Mode of transmission varies across Europe with 40% of transmission occurring in MSM in Western Europe, 29% due to heterosexual sex and only 8% in IVDU. In Central Europe these figures are 30%, 40% and 13% respectively. In Eastern Europe 42% of new infections result from transmission due to heterosexual sex, only 0.4% amongst MSM, and 57% in IVDU [41]. In Europe overall, the number of HIV reports among MSM increased by 39% between 2003 and 2007 [42]. A large proportion of new diagnoses in Europe which were due to heterosexual transmission acquired the infection in a country with a generalised epidemic (mostly sub-Saharan Africa, the Caribbean, and Asia) [42].

All studies show that unprotected anal intercourse is more risky than vaginal sex and that receptive sex is riskier than active sex [43]. A study of African HIV-1 serodiscordant couples (infected partner not treated) showed the unadjusted per-act transmission rate to be 0.0019% for male-to-female transmission and 0.0010% for female-to-male transmission during heterosexual intercourse [44]. The risk of transmission in receptive anal intercourse appears to bear the highest risk with per-act transmission rates of 0.04 - 3.0% [43]. The fragility of rectal muscoa compared with the female genital tract may account for this. It seems that the stage of the disease in the infected partner is important, in that HIV-1 transmission per heterosexual coital act follows a U-shaped curve, being highest during primary infection, lower during the asymptomatic phase, and increasing with advancing disease [45]. This may be due to differing amounts of the virus in the blood over the course of increase, which is usually measured as plasma viral ribonucleic acid (RNA)-load. Indeed, each $\log_{10}$ increase in viral RNA-load has been shown to increase the per-act risk of transmission by 2.9-fold (95% confidence interval, 2.2-3.8) [44]. Other factors influence transmission rates via the various modes; for example, heterosexual sexual transmission rates in low-income countries are thought to be four to ten times higher due to as yet undetermined reasons [46]. The presence of genital herpes simplex virus (HSV)-2 infection has been shown to be a risk factor for HIV acquisition [47], as has active syphilis [48] and gonorrhea [49] amongst other sexually transmitted infections (STI). Such STI have been shown to facilitate HIV transmission by increasing both infectivity [50] and susceptibility to infection [45]. STIs which cause genital ulceration are thought to increase transmission as a result of disturbing the integrity of the genital mucosa [51]. Pregnancy also appears to be associated with increased male to female transmission of HIV [52].

Mother-to-child transmission of HIV, which can occur during pregnancy, delivery or breastfeeding, accounts for the vast majority of HIV infection in children worldwide [1]. Current prevention strategies, widely employed in developed countries (see Section 1.8), reduce mother-to-child transmission from around 25% to as low as 1% [53]. However, huge numbers of children continue to be infected in sub-Saharan Africa; UNAIDS (the Joint United Nations Programme on HIV/AIDS) estimate 330000 (190000 – 460000) were infected in 2009 alone [54].

Subtype B isolates appear to be spread mostly by homosexual contact and in IVDU, while subtype C and CRF01_AE are more closely associated with heterosexual contact. Some studies have found decreased perinatal transmission of subtype D compared with other subtypes [55, 56], whereas yet other studies have found no difference [57, 58] or high rates of subtype C transmission compared with subtypes A or D [59]. It has been shown that subtype C gives rise to high viral RNA-load and higher levels of vaginal shedding than subtype A or D [57]. This

may be related to the fact subtype C viruses have three NFκB sites in comparison to one or two in other subtypes [60]; during immune activation, these sites make HIV-1 more responsive to cytokines such as tissue necrosis factor (TNF)-α that enhance virus production. The high viral RNA-load and vaginal shedding has been proposed to be the reason for the particularly rapid epidemic spread of subtype C HIV-1, although other factors co-existing in populations affected by this subtype may alternatively account for its rapid spread.

1.2.2 HIV-1 Prevalence and Incidence

UNAIDS estimate that at the end of 2010 34 million people were living with HIV worldwide [1]. Sub-Saharan Africa is by far the region most affected by HIV with 68% of all people infected with HIV worldwide living here; prevalence is estimated to be approximately 5%. Prevalence is similar in East Africa, whilst in West and Central Africa prevalence is lower at around 2% or under. The Caribbean bears the second highest prevalence of HIV at 0.9%. Latin America is also seriously affected with a lower but still significant prevalence of 0.4%. In South and South-East Asia prevalence is low, 0.3%, compared with Africa, but the region's significant total population means that a huge number of people are living with HIV. Generally, prevalence in the Middle East and North Africa is thought to be low; however, reliable data in some of these regions is not available. In Eastern Europe and Central Asia prevalence is 0.9%, in Western and Central Europe it is 0.2% and in North America 0.6% [1].

There were an estimated 2.7 million new HIV infections in 2010, with 1.9 million of these occurring in sub-Saharan Africa [1]. Fortunately, gains appear to be being made as new infections are thought to be down 21% overall from the peak of the global epidemic in 1997, with incidence falling in 33 countries including those in sub-Saharan Africa, the Caribbean and Asia. Yet UNAIDS report that between 2001 and 2010, there has been a 250% increase in the total number of HIV infections reported in Eastern Europe and Central Asia, of which 90% are accounted for by the Russian Federation and the Ukraine. This increase appears to be due to infections in IVDU and as a result of heterosexual transmission. The incidence in North America, Western and Central Europe has overall stayed static since 2004 despite the high standards of health care in these regions and access to ART [1].

In the United Kingdom, the Health Protection Agency reports a year on year decline in new HIV infections with an estimated 6150 new diagnoses in 2011 compared with the peak of 7824 in 2005 [61]. This represents a decreasing incidence of infection in heterosexual individuals but

an increasing incidence amongst MSM. About 44% of people newly infected with HIV in 2007 in the U.K. had acquired it abroad, mainly in sub-Saharan Africa [62].

1.2.3 Global distribution of HIV-1 Subtypes

Whilst groups N and O have remained confined to part of West Central Africa (Gabon, Cameroon and Equatorial Guinea), group M is responsible for the worldwide pandemic, representing more than 95% of global virus isolates. The global distribution of each group M subtype and CRF has its own distinct geographic pattern. Worldwide 48% of infections are due to subtype C, 12% subtype A, 11% subtype B, 8% CRF02_AG, 5% CRF01_AE, 5% subtype G and 2% subtype D [24]. Here is a summary of the predominant subtypes / CRFs (see also Figure 1.2) [24] :

- Subtype C is almost the exclusive subtype causing the epidemic in South Africa, Ethiopia and India, but is also circulating in East Africa, South and South-East Asia.
- Subtype A is the dominant subtype in East Africa and is also widespread in Eastern Europe and Central Asia. It is also found in West and Central Africa and South and South-East Asia.
- Subtype B dominates in North America, the Caribbean, Latin America, Western and Central Europe and Australia. It also circulates in North Africa, South and South-East Asia, Eastern Europe and Central Asia.
- CRF02_AG is a complex mosaic of subtype A and G sequences and circulates widely in West Africa and to a lesser extent in Central Africa, the Middle East and North Africa.
- CRF01_AE is found mostly in South and South-East Asia, East Asia and to a lesser extent in Central Africa.
- Subtype G is seen mostly in West and Central Africa.
- Subtype D causes a large number of infections in East and Central Africa, where it has been co-circulating with subtypes A and C[24].

As mentioned, in Western and Central Europe subtype B is dominant; however, other subtypes are increasing in prevalence [63]. Indeed, all major subtypes and many CRFs and URFs are detected. In the U.K. 74.9% of isolates which have undergone sequence analysis are subtype B, 10.0% are subtype C, 6.4% are subtype A and 1.8% are subtype CRF02_AG together with many other less frequent subtypes [64]. This appears to reflect the high proportion of HIV-1 infected individuals in the U.K. who are MSM since most men are infected with subtype B

virus, although the proportion infected with non-B subtypes is increasing [65]. In contrast, data from England and Wales show that subtype C was the most prevalent subtype amongst infected heterosexuals [66]. London is a particular melting pot with the above mentioned strains represented, together with subtypes F, G, H CRF06_cpx, CRF11_cpx, CRF12_BF, CRF14_BG and many other CRFs and URFs [67].

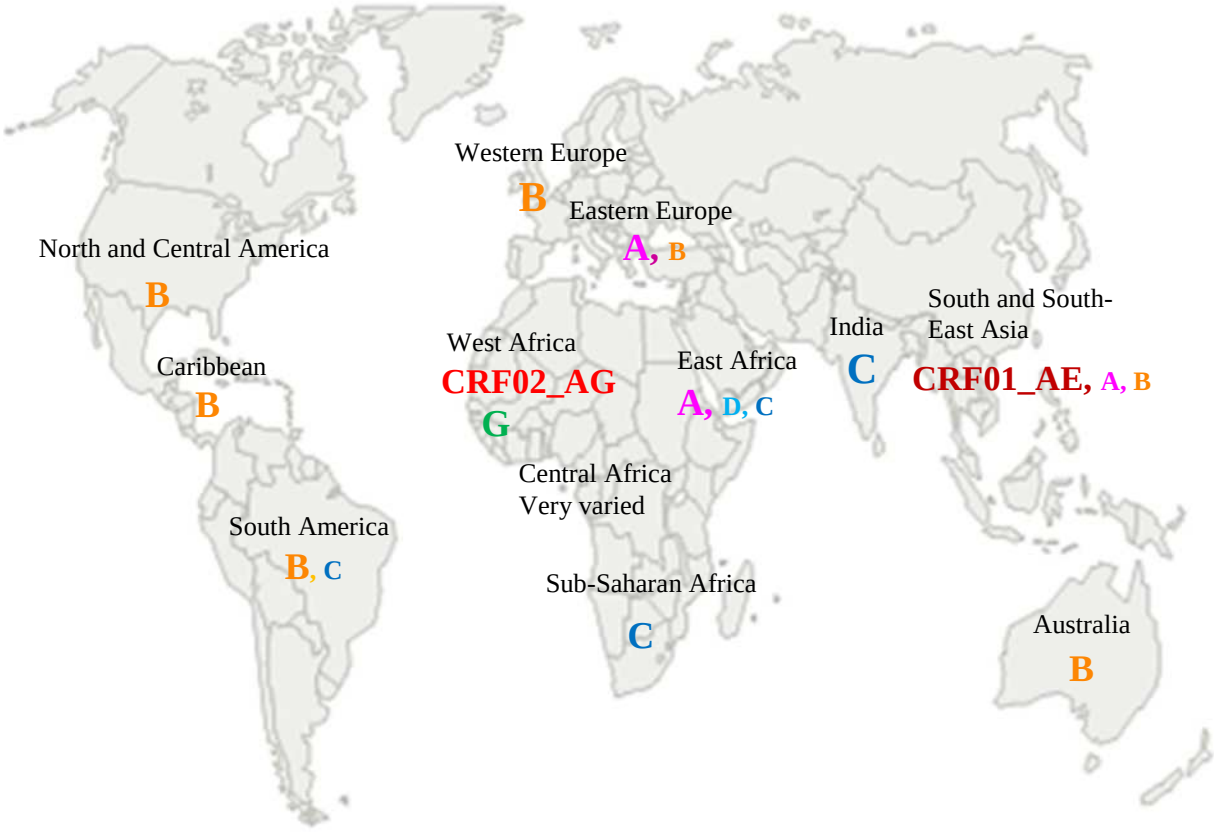


Figure 1.2 Geographical distribution of HIV-1 subtypes. Genetic forms predominant in an area are shown in letters of larger size. The greatest diversity is found in Central Africa where all subtypes and many CRFs and URFs are represented. Data taken from Hemelaar *et al.* [24].

1.3 HIV-1 Genomic Organisation and Structure

The organisation of the HIV-1 genome together with encoded proteins are shown in Figure 1.3.

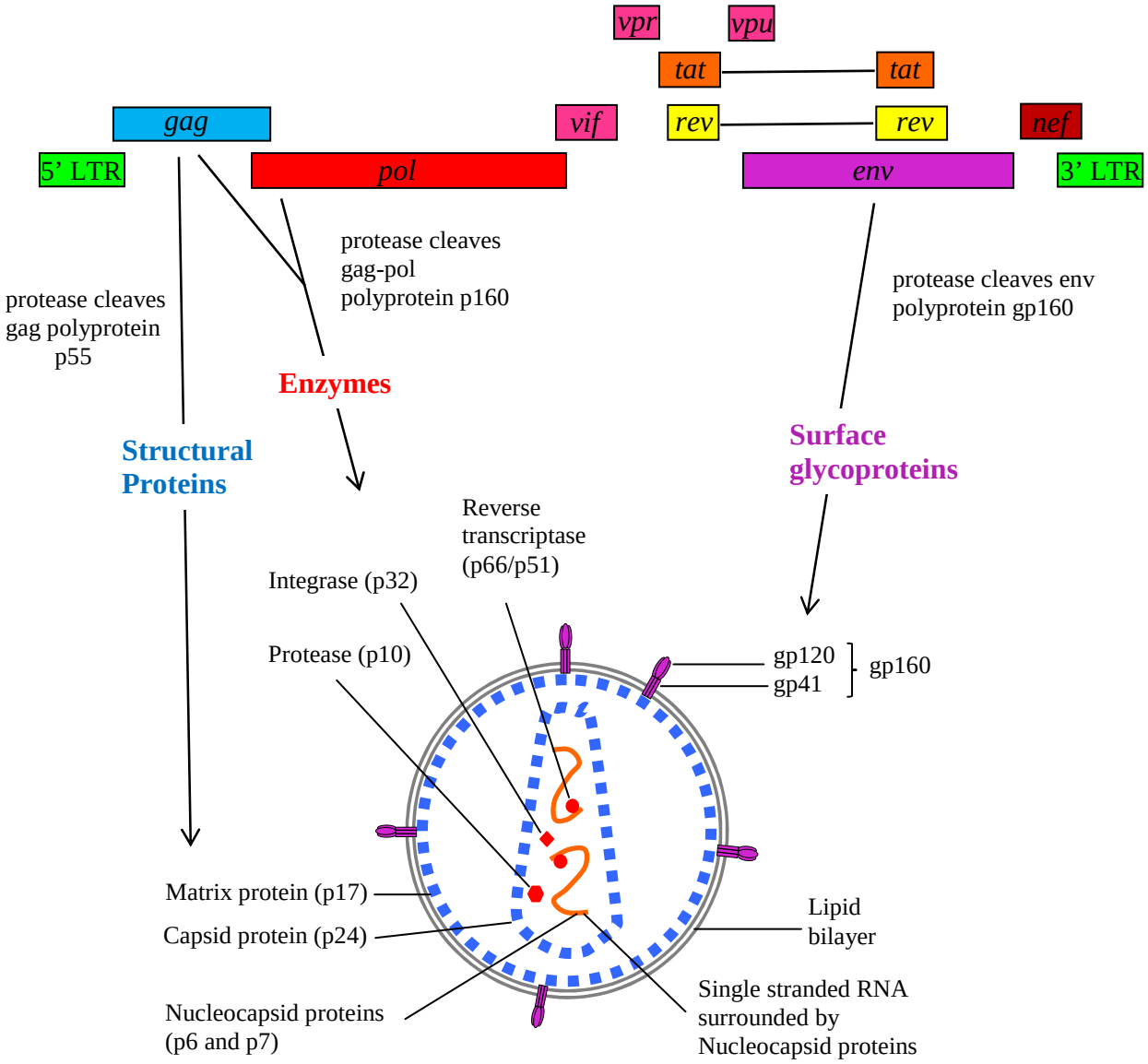


Figure 1.3 Genomic organisation and viral structure of HIV-1. Adapted from [68]. This figure is schematic and simplified for clarity.

The HIV-1 virion has a diameter of 100-120nm with spherical morphology and is surrounded by a lipoprotein membrane. The envelope glycoprotein (gp), Env, is integrated into this membrane and is composed of a trimer of the external (gp120) and transmembrane (gp41) subunits [69]. The matrix protein p17 is anchored to the internal face of the lipoprotein membrane. Inside this is a conical shaped capsid (composed of protein p24) which contains two genomic single-stranded positive sense RNA strands (diploid genome), each around 9.7 kilobases and tightly bound to the nucleocapsid proteins p6 and p7, and the viral enzymes [70, 71].

As with other retroviruses, the HIV-1 genome encodes three main genes: *gag* (group-specific antigen), *pol* (polymerase) and *env* (envelope) arranged as 5' LTR-*gag*-*pol*-*env*-LTR 3'. The LTR (long terminal repeat) regions represent the two end parts of the viral genome that are connected to the deoxyribonucleic acid (DNA) of the host cell after integration (see the next Section) and do not encode for any viral proteins. LTR has a transcription-promoter function and contains regions important in reverse transcription [72] and genomic RNA dimerisation (non-covalent joining of the two RNA strands) [73]. The genomic organisation of HIV-1 is extremely efficient, employing all three reading frames and multiple splicing to encode 15 proteins from a single mRNA [74].

The *env* gene codes for the polyprotein gp160 which subsequently undergoes proteolytic cleavage to form gp120 and gp41. The *gag* gene codes for the gag polyprotein (p55) which is cleaved to form the structural proteins p17, p24, p6 and p7. A gag-pol polyprotein (p160) is cleaved to form the enzymes reverse transcriptase (RT, composed of two subunits p66 and p51), integrase (p32) and protease (p11). In addition, HIV-1 encodes regulatory proteins 'trans-activator of transcription' (Tat) and 'regulator of virion expression' (Rev) and accessory proteins 'negative regulatory factor' (Nef), viral infectivity factor (Vif), 'viral protein R' (Vpr) and 'viral protein U' (Vpu) [74].

1.4 HIV-1 Life Cycle

The HIV-1 life cycle is shown in Figure 1.4.

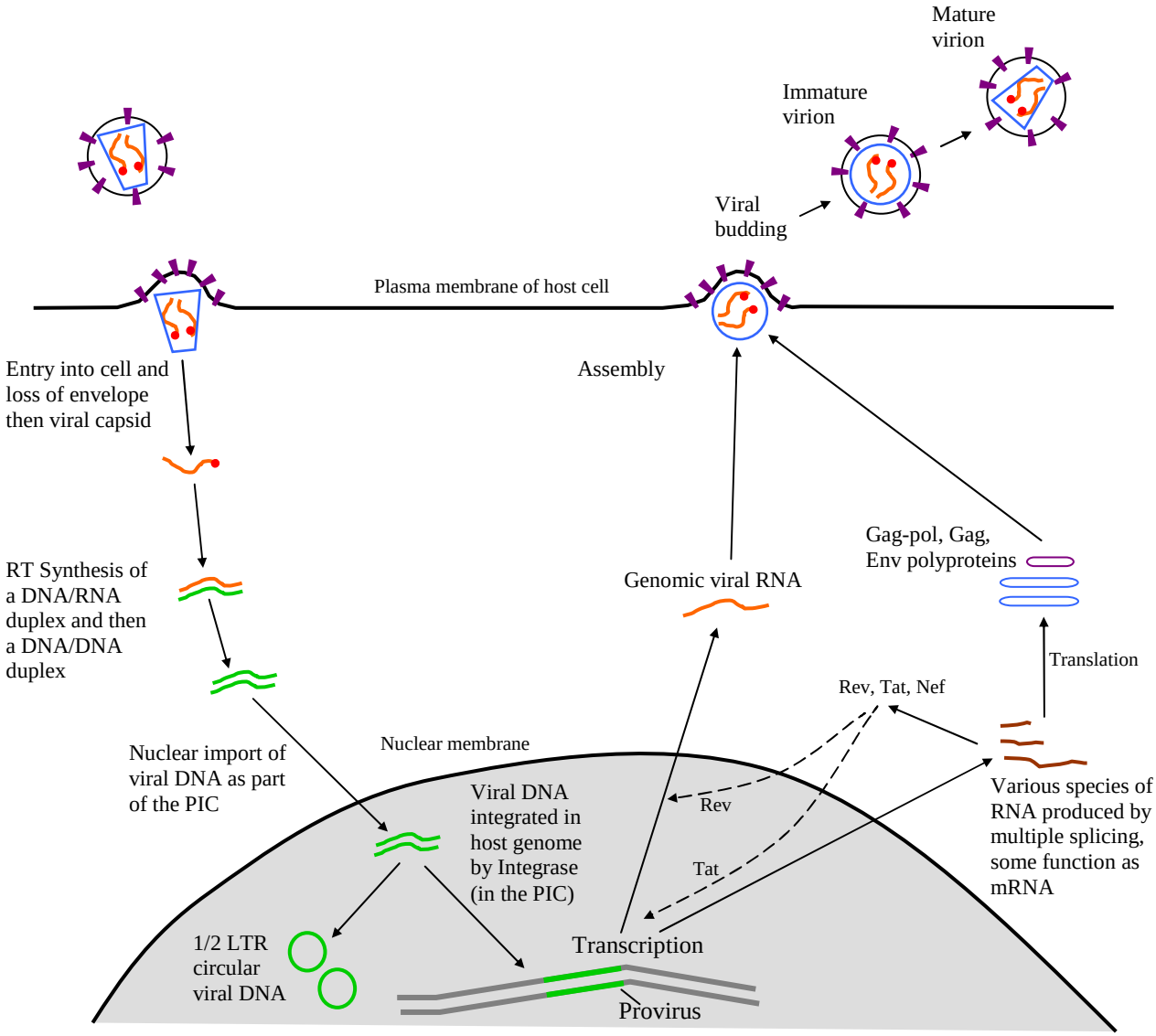


Figure 1.4 Life cycle of HIV-1.

HIV-1 cell entry requires cognate recognition of gp120 with the cell surface CD4 molecule on the surface of T-cells, monocytes, macrophages and dendritic cells. A co-receptor is also required for attachment to cells and most viruses use one of two co-receptors, C-C chemokine receptor type 5 (CCR5) or C-X-C chemokine receptor type 4 (CXCR4) [75, 76]. CCR5 and CXCR4 are chemokine receptors involved in regulating the inflammatory response. As such, the expression patterns of CD4 and the co-receptor are major determinants of HIV-1 tropism. Based on this tropism, HIV-1 strains can be broadly divided into two categories, those using the CCR5 co-receptor (R5 viruses) and those which use the CXCR4 co-receptor (X4 viruses); also some isolates may use either [77]. CXCR4 is preferentially expressed on naïve, unactivated T-cells, whereas CCR5 is expressed on the activated effector memory T-cell subset, and also on cells of the dendritic cell and macrophage lineage. A large number of other chemokine receptors such as CCR3 can also act as a primary or secondary attachment sites but are not commonly involved in infection [78].

After binding CD4 there is a significant conformational change in gp120 which in turn leads to conformational changes in gp41, resulting in fusion between the viral and cellular membranes and release of the viral capsid into the cell cytoplasm [69].

Subsequently, a preintegration complex (PIC) is formed, which is a nucleoprotein complex of viral proteins (including Vpr, p17 and integrase), host proteins and the two strands of viral RNA [79]. Within the HIV-1 PIC, viral RNA is reverse-transcribed into complementary double-stranded DNA (dsDNA) by the viral RT (also referred to as the RNA-dependent DNA polymerase). The viral RT is particularly error-prone leading to frequent mutations and thus contributing to the generation of the viral quasispecies [80].

A single susceptible cell may be infected with two different HIV-1 isolates (different subtypes in a dually infected individual, or differing quasispecies). Some progeny virions released by the cell will contain two viral RNA strands which are not identical (from each isolate), referred to as a heterozygous virus. When the heterozygous virus infects a new cell, retroviral recombination occurs when the RT enzyme switches between these co-packaged RNAs during reverse transcription (‘template switching’) [81]. Retroviral recombination thereby enables beneficial mutations from different viruses to be combined in a single virion and deleterious mutations may effectively be lost.

The PIC carrying the newly synthesised HIV-1 dsDNA enters the nucleus. The viral integrase catalyses the integration of the viral dsDNA into the host cell genome; the integrated viral DNA is referred to as the ‘provirus’. The site was once thought to be entirely random [82], but

further studies suggest integration may be at a transcriptionally active area of the chromosome [83, 84]. Integration at such a site might be important for efficient transcription from the viral genome. Other studies have been able to link the integration site with viral RNA-load; in patients with low viral RNA-load the provirus was found to be integrated predominantly in GC-rich regions, while in patients with high viral RNA-load the integration of viral genomes was in GC-poor regions [85]. The provirus is replicated as part of mitotic cell division and may persist in this form for long periods and through many rounds of division.

A number of unintegrated viral dsDNA species are also generated and are considered terminal products of the reverse transcription process which are unable to sustain replication themselves [86, 87]. These unintegrated forms are linear or circularised and arise when the viral dsDNA fails to integrate into the host genome: a 2-LTR circle is formed when the ends of the linear dsDNA join; a 1-LTR circle may result from homologous recombination between the two LTRs in a 2-LTR circle; or dsDNA may remain unintegrated in the linear form [87]. After integration, cellular transcription factors activate viral gene transcription from the provirus, producing numerous species of mRNA by multiple splicing [88]. About half of these are estimated to be full-length RNA which function as mRNA and genomic RNA for secreted virions. Other mRNA transcripts encode Tat, Rev, and Nef (see the next Section).

Virions emerge from the infected cell by budding as immature non-infectious particles containing uncleaved Gag polyproteins [87, 89]. Gag is eventually cleaved by the viral protease, leading to a further assembly stage forming the mature cone-shaped capsid with generation of mature infectious virus particles. Release of new virions often occurs at the site of contact between an infected and uninfected cell with the formation of a ‘virological synapse’ [90]. In this way virus may spread directly to the uninfected cell, without exposure to the humoral immune system [91].

1.4.1 HIV-1 Regulatory and Accessory Proteins

Tat is a transcriptional transactivator for the LTR promoter which vastly increases the level of transcription from the provirus. It does this by preventing premature termination of provirus transcription through binding to the 5' ends of nascent HIV-1 transcripts (the trans-activating response element, TAR) enabling the production of full-length HIV-1 transcripts [92]. Tat is also actively secreted by infected cells and is endocytosed by uninfected cells. It strongly increases the expression of HIV-1 co-receptors, thus favouring HIV-1 infection of the uninfected cell, and also modulates the production of immunoregulatory cytokines, thereby

contributing to immunosuppression [93]. Extracellular Tat is also thought to induce T-cell apoptosis [94].

Rev has been shown to mediate the nuclear export of incompletely spliced RNA transcripts so that the structural proteins and RNA genome can be produced [95]. In the absence of the *rev* gene, the host RNA splicing machinery in the nucleus quickly splices the RNA so that only Rev, Tat and Nef can be produced.

Nef is a major virulence factor which contributes to maintaining high viral RNA-loads and $CD4^+$ T-cell depletion by inducing Fas-mediated apoptosis of uninfected bystander $CD4^+$ T-cells [96]. It also leads to endocytosis and degradation of cell surface molecules such as CD4 and class I human leukocyte antigen (HLA), with the result that infected cells might evade the host cell-mediated immune response [88, 97]. The pivotal role of Nef is highlighted by the fact that SIV with deletion in the *nef* gene is attenuated and can serve as a live attenuated virus vaccine [98].

Replication in non-dividing cells such as macrophages is promoted by Vpr, whereas its role appears less essential in other target cells [99]. It also induces cell cycle arrest in G2 and apoptosis in proliferating cells and so may significantly contribute to $CD4^+$ T-cell depletion [100]. The function of Vif and Vpu are covered in the next Section.

1.4.2 Host Restriction Factors which Target HIV-1 Replication

Specific host proteins have evolved to limit retroviral replication. Apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (APOBEC3G) and related family members are potent retroviral restriction factors. They function by deaminating cytidine to uridine resulting in hypermutated viral DNA with frequent stop codons [101]. Vif acts to inhibit APOBEC3G activity by connecting the enzyme to a polyubiquitous complex leading to its proteosomal degradation [102]. Another intracellular factor, tripartite motif protein 5 alpha (TRIM5α) in rhesus macaque monkeys has been shown to cause rapid disassembly of the viral capid with the result that reverse transcription is not completed; this effect is not seen with human TRIM5α [103]. Tetherin, also produced as part of the innate immune system, exerts antiviral activity by opposing the release of progeny virions at the surface of infected cells and is opposed by Vpu [104]. Further aspects of the innate immune system are discussed in Section 1.7.4.

1.5 Natural History of HIV-1 Infection

Figure 1.5 summarises the typical natural history of untreated HIV-1 infection over time.

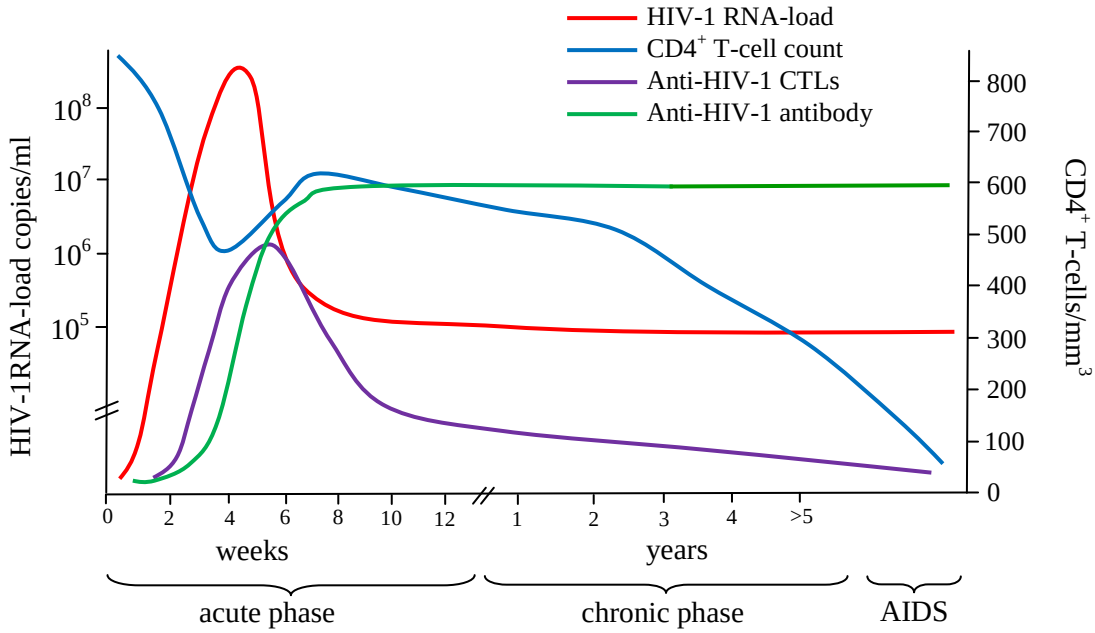


Figure 1.5 The natural history of HIV-1 infection. HIV-1 RNA-load, peripheral blood $CD4^+$ T-cell count, HIV-1-specific cytotoxic T lymphocyte (CTL) and antibody response over time following primary infection.

1.5.1 Acute HIV-1 Infection

The most common mode of HIV-1 infection is sexual transmission through the genital mucosa. It is likely that the initial infection by HIV-1 occurs in most patients by a single virus and that these 'founder' viruses tend to use the CCR5 as a co-receptor [105]. This would explain why persons who are homozygous for a deletion in CCR5 (CCR5Δ32) are relatively resistant to infection with the usual HIV-1 R5 strains [106].

Much understanding of the early events during HIV-1 infection has been obtained by the study of SIV infection of rhesus macaque monkeys, since it recapitulates the pathogenic effects of HIV-1 infection of humans [107]. Some such studies suggest that immunophenotypically resting $CD4^+$ T-cells in the genital mucosa are the predominant cell type infected during heterosexual transmission, rather than activated cells as had been believed [108]. Dendritic cells also appear to play a role whereby they capture HIV-1 at the mucosal surface via C-type lectins, and then traffic the virus to lymph nodes and present it there to activated T-cells [109]. The virus is then thought to remain localised in genital/rectal mucosa and draining lymph nodes

for around 10 days [110]. Immune activation in response to the infection leads to more activated $CD4^+$ T-cells, in which efficiency of virus production is greater, thus enhancing dissemination of infection [111, 112]. Virus then spreads via the blood to other lymphoid tissue [113]. At these sites it replicates profusely and the level of free virus in the blood rises exponentially to reach a peak, often exceeding 10^6 copies/ml 21–28 days after infection [114]. A mononucleosis like syndrome may occur during this phase [115]. Routine laboratory assays may detect infection with assays which detection the virus in the blood becoming positive first, following by serological tests (se Section 1.6.1).

Studies in acute SIV [108, 116, 117] and HIV-1 infection [118], suggest that during peak viral RNA-load there is a profound depletion of $CD4^+$ T-cells (particularly effector memory $CD4^+$ T-cells) in extra-lymphoid effector sites, particularly in the GI (gastrointestinal) tract lamina propria [108, 118-120]. The GI tract lamina propria is particularly rich in $CD4^+CCR5^+$ effector memory T-cell targets for infection [121, 122]. $CD4^+$ T-cell populations in blood and secondary lymphoid tissues (including the gut-associated lymphoid tissue, GALT) are not as dramatically affected at this early stage because $CD4^+CCR5^+$ effector memory T-cells are only minor components of the overall $CD4^+$ T-cell compartment in these sites [118].

Virus levels then fall over several months, rapidly at first, until a stable level is reached during the asymptomatic phase preceding progression to AIDS, and can vary among individuals by as much as 4 to 5 logs [123]. The extremely fast decline in viral RNA-load upon initiation of ART [124] suggests that the vast majority of this free form of the virus in plasma is produced by recently infected cells. The cellular HIV-1 DNA-load, although not routinely measured, is a marker of viral replication and has been shown to follow a similar downward trend following acute infection as the viral RNA-load [125].

1.5.2 Chronic HIV-1 Infection

After the acute phase of infection and viral RNA-load set point is achieved, an asymptomatic phase ensues. During this phase, HIV-1 replication largely evades immune control due in part to the presence of latently infected cells [126]. HIV-1 latency is defined as either pre-integration or post-integration latency. Pre-integration latency occurs where unintegrated HIV-1 DNA exists in the PIC in the cytoplasm and decays over time [127]. Post-integration latency refers to a state whereby the virus has integrated into the host genome but is transcriptionally silent; these cells are typically long-lived [128]. The majority of cells exhibiting post-integration latency are resting memory $CD4^+$ T-cells [129]. It has been proposed that latently infected cells result from HIV-1 DNA integrating into an activated cell

which subsequently returns to a resting state [130]. However, numerous alternate mechanisms have also been proposed including mutations in Tat [131], absence of cellular transcription factors [132], gene regulation by microRNAs [133] and proviral integration into transcriptionally silent sites [134].

Macrophages are thought to play an important role in HIV-1 latency. They are long-lived, terminally-differentiated tissue-resident cells which act to assist in clearance of pathogens and apoptotic cells, and also have a role in antigen presentation to T-cells [135]. They express the required cell surface receptors for HIV-1 infection and are thought to be the primary non T-cell reservoir for HIV-1 [136], demonstrating long-lived productive HIV-1 infection, often without obvious cytopathic effects [137]. Mature virus particles have been shown to remain trapped within surface invaginations giving the appearance of an intracellular budding compartment [138, 139]. It is thought that virus at this site is protected from the immune clearance. It has been proposed that virions could be released from these invaginations over extended periods of time [139]. Furthermore, infected macrophages are thought to provide an ongoing source of infectious virus which is transmitted to susceptible $CD4^+$ T-cells via a virological synapse [140]. Growing evidence indicates an important role of infected macrophages in a variety of HIV-1-associated diseases, including neurocognitive disorders [141].

During the asymptomatic phase there is a gradual depletion of circulating $CD4^+$ T-cells. It has been documented that in the blood a fast initial decline (5.2 cells/mm^3 per week) of $CD4^+$ T-cells is seen over the first 5-6 months, followed by a slower rate of decline (1.9 cells/mm^3 per week) [142]. $CD4^+$ T-cells play a central role in the adaptive immune response to viral infections most importantly as they are critical for the maintenance of effective CTL function [143], but also through their capacity to help B-cells make antibodies, enhance microbicidal activity of macrophages and recruit cells from the innate immune system through production of cytokines and chemokines. Therefore the ongoing depletion of $CD4^+$ T-cells over the asymptomatic phase eventually results in immune dysfunction and the development of AIDS. AIDS is defined as a $CD4^+$ T-cell count of <200 cells/mm^3 or an AIDS defining opportunistic infection or neoplasm [144]. The average time from infection to symptomatic AIDS is around 10 years [145].

As infection progresses, there is an increased prevalence of X4 viruses with decreasing $CD4^+$ T-cell count [146]. However, X4 viruses emerge in only about half of the patients who progress to AIDS [147]. Given the long interval between infection and emergence of X4 variants it is plausible that their emergence is a consequence and not cause of disease progression. Indeed, as mentioned in Section 1.1 subtype D has been associated with increased pathogenicity, which

might result from the fact that phenotypic assays have shown X4 viruses to be particularly prevalent in subtype D infection [148, 149], thus suggesting this subtype may have a greater propensity to switch to CXCR4 usage.

1.6 Clinical Management of HIV-1 Infection

1.6.1 Laboratory Diagnosis and Monitoring of HIV-1 Infection

The laboratory diagnosis of HIV-1 infection is usually made by detection of virus specific antibodies by enzyme-linked immunosorbent assay. Patients may be tested based on clinical suspicion or as part of a screening programme, e.g. during pregnancy. Antibodies to HIV-1 typically become detectable around 4 weeks after infection [150]. In addition to antibody, the British HIV Association (BHIVA) recommends that this initial assay simultaneously detects p24 antigen (the capsid protein) as these fourth generation assays may be positive 1 - 2 weeks earlier than antibody only assays [144].

If positive, there is a requirement for the test to be confirmed with two further assays, one of which should distinguish HIV-1 from HIV-2. The diagnosis of primary HIV-1 infection before seroconversion depends on detection of either p24 or a detectable viral RNA-load in plasma (positive approximately seven days before p24 assays) [151]. Several commercial HIV-1 RNA-load assays are available which are highly automated, typically using real-time polymerase chain reaction (PCR) methods [152]. They are able to detect all subtypes within group M HIV-1 (some also detect group O) [152]. However, the genetic diversity of HIV-1 means that viral RNA-load is occasionally underestimated using a single assay [153]. In particular the Cobas AmpliPrep/Cobas TaqMan HIV-1 assays were shown to underestimate viral RNA-load in CRF02_AG subtype infection [154]. HIV-1 subtypes are usually determined by sequence alignment and phylogenetic analysis with reference strains.

In the U.K. and other Western countries HIV-1 infection is usually diagnosed whilst in the asymptomatic phase. The duration of this phase is variable and therefore the patient requires 3-6 monthly follow-up visits where blood is taken for viral RNA-load and $CD4^+$ T-cell count monitoring. There remains some debate regarding whether absolute $CD4^+$ T-cell count or $CD4^+$ T-cell percentage (percentage of lymphocytes that are $CD4^+$) is a more reliable marker of immune status in HIV-1 infected individuals. General consensus and most treatment guidelines tend to use absolute $CD4^+$ T-cell count [144].

During the clinic visit evidence of opportunistic infections or neoplasm is determined by thorough history taking and physical examination. Assessment is made of any non-AIDS-defining morbidities including cardiovascular, metabolic, renal and bone diseases. The presence of commonly co-infecting organisms is determined using further blood assays: toxoplasma, syphilis, hepatitis B and C [144]. Guidelines suggest that once $CD4^+$ T-cell counts fall below 450 cells/mm^3 the patient should be seen more frequently in order to monitor count drops to 350 cells/mm^3 which would necessitate the initiation of treatment [155].

1.6.2 Treatment of HIV-1 Infection

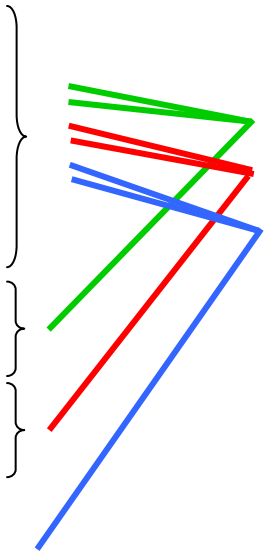
Treatment of HIV-1 with ART has significantly prolonged the time to both AIDS development and death in those infected [156], although significant challenges exist regarding toxicity, drug-drug interactions and patient compliance. Generally there is agreement that there is benefit in beginning treatment in asymptomatic patients with $CD4^+$ T-cell counts below 350 cells/mm^3 [144]. There is debate regarding whether treatment is beneficial where the count is 350-450 cells/mm^3 [157].

Many of the newer potent, once-daily regimens are now well tolerated and make durable suppression of viral RNA-load achievable for most patients. To be effective, treatment must suppress viral RNA-load to below the limits of detection of current standard assays (typically 40 -50 copies/ml) [144].

The targets of commonly used ART agents are reverse transcription (nucleotide/nucleoside RT inhibitors, NRTI, and non-nucleoside RT inhibitors, NNRTI), protein cleavage (protease inhibitors), entry (fusion inhibitors) and integration (integrase inhibitors). Examples of these are shown in Table 1.1. The most important factor responsible for failure of ART is drug resistance and it is this which continues to drive the generation of new drugs. The development of the protease inhibitor, ritonavir, was a particularly significant advance. It can be used in combination at low dose to significantly boost plasma levels of other protease inhibitors as it is a potent inhibitor of cytochrome P450 3A4 (CYP3A4), thus avoiding the toxicities of using it at therapeutic dose and yet providing a much higher barrier to failure due to resistance [158]. A new class of drugs is the CCR5 co-receptor antagonists, which bind to the CCR5 co-receptor of the susceptible $CD4^+$ T-cell, preventing viral entry [159]. A further group, called maturation inhibitors, specifically inhibit the final stage in Gag processing which causes an infected cell to produce virions which are non-infectious [160]. Highly active ART (HAART) refers to the combination of drugs which are typically given (often three drugs) since combining in this way enhances efficacy; common combinations are also shown in Table 1.1 [144].

Table 1.1 Common anti-retrovirals and HAART combinations.

Drug Group	Common drugs within group
Nucleotide/nucleoside RT inhibitors	Abacavir
	Emtricitabine
	Zidovudine
	Lamivudine
	Tenofovir
Non-nucleoside RT inhibitors	Efavirenz
	Nevirapine
Protease inhibitors	Darunavir (boosted with ritonavir)
	Atazanavir (boosted with ritonavir)
Fusion inhibitors	Enfuvirtide
Integrase inhibitors	Raltegravir
CCR5 antagonists	Maraviroc



BHIVA currently recommends HAART containing two nucleos(t)ide reverse transcriptase inhibitor plus one of the following: a ritonavir-boosted protease inhibitor, an NNRTI or an integrase inhibitor [144]; these combinations are shown with the coloured lines on the right.

New approaches to drug treatment include the induction-maintenance approach whereby HAART (induction), may be followed by a maintenance phase using one or two drugs [161] and intermittent therapy [162] but have been shown to be inferior to continuous HAART.

A sharp decrease in viral RNA-load is usually seen quickly after starting treatment, with most patients achieving undetectable viral RNA-load by week 12 to 24 [144]. If this does not happen concern is usually raised about treatment adherence or viral resistance to treatment [163]. Despite this rapid decrease in viral RNA-load, which suggests suppressed viral replication, some studies have shown that *env* sequences continue to evolve suggesting that replication is in fact ongoing [164]. In keeping with this, some patients with sustained undetectable viral RNA-load occasionally experience intermittent episodes of detectable viral RNA-load (blips) [165]. Following the suppression of viral RNA-load HIV-1 DNA is also seen to decline [86].

An increase in total peripheral $CD4^+$ T-cell counts follows the decrease in viral RNA-load following ART initiation. An initial increase of 50-100 cells/mm^3 may be seen followed by a more gradual increase [166]. The initial increase is thought to be due to redistribution of $CD4^+$

T-cells from lymphoid tissues into the circulation [167]. The subsequent gradual increase is thought to be due to an increased production of new $CD4^+$ T-cells [164]. Treatment is also seen to reduce levels of immune activation which is thought to be important in disease pathogenesis (see Section 1.9.1) [166].

1.7 Immune Response to HIV-1 Infection

As with other viral infections, infection with HIV-1 elicits both cellular and humoral adaptive immune responses together with the innate immune system.

1.7.1 Cell-Mediated Immunity

Focusing first on cell-mediated immunity, HIV-specific T-helper cells ($CD4^+$ T-cells) assist cytotoxic T lymphocytes (CTLs, specialised $CD8^+$ T-cells) to kill HIV-infected cells, which present viral peptides associated with class I HLA molecules [168]. CTLs are the effector arm of the cell-mediated immune system for which the main function is to detect and destroy cells infected by intracellular pathogens. CTLs target these pathogens by two main effector mechanisms: lysis of the target cell *via* Fas–FasL interaction or lysis by the delivery of granzymes and perforin to the target cell. Of these, the latter mechanism is the most rapid and is usually the most important in anti-viral defence [168]. Most HIV-1 infected individuals have very high numbers of virus-specific CTLs compared with the number of antigen-specific CTL produced during other viral infections [169]; it is not completely understood why viral replication continues in spite of this strong CTL response. Nevertheless, HIV-specific CTL appear to play a crucial part in control of HIV-1 based on several observations. During primary infection, there is a temporal relationship between HIV-1 and the virus-specific CTL response since these CTL appear in the blood just before viral RNA-load peaks. This population then expands and contracts as viral RNA-load falls [170, 171]. Work with SIV-infected rhesus macaques demonstrated that during primary infection, viral RNA-load was not controlled in monkeys depleted of $CD8^+$ lymphocytes (including T-cells and natural killer (NK) cells but predominantly CTLs) using anti-CD8 monoclonal antibody [172]. Therefore, HIV-specific CTLs appear crucial in bringing the high level viraemia during primary infection under control.

Whilst in an acute viral infection, the efficiency of the CTL response is in general correlated with the frequency of virus-specific CTL, this association is less clear for chronic viral infections [173]. CTL proliferate in response to antigen, so therefore the frequency of CTL may be both a cause and an effect of the viral RNA-load [173, 174]. Indeed, work with SIV-

infected rhesus macaques shows that in chronic infection CTL frequency is the cause of low viral RNA-loads, since depleting these cells during chronic infection led to a significant rise in viral RNA-load that was again suppressed when SIV-specific $CD8^+$ lymphocytes were reinstated [172]. However, interpretation of the fact that there is an inverse relationship between frequency of HIV-specific CTL activity and viral RNA-load [175, 176] is more difficult. Two competing explanations are that higher CTL frequency leads to control of viral RNA-load or higher viral RNA-load leads to greater HIV-induced immunosuppression leading to CTL loss. The former rather than the latter appears to be the case, since when HIV-infected patients with high viral RNA-load are treated with ART, thus decreasing viral RNA-load, HIV-specific CTLs are also seen to decrease [177-179]. However, the situation is likely to be more complicated than this given positive and negative correlations may be found at the same time in the same individual between viral RNA-load and CTL specific to different HIV-1 epitopes [180]. A potential explanation might be that 'driver' CTL specific to certain epitopes control the viral replication and hence viral RNA-load, whereas 'passenger' CTL specific to other epitopes proliferate in response to viral antigen without themselves having an effect on viral RNA-load [181].

Further to the size of the CTL pool is the efficiency or 'quality' of the CTL response. It has been demonstrated that HIV-specific $CD8^+$ T-cells from viraemic patients are often dysfunctional. This is demonstrated by low levels of perforin expression [182, 183], limited *ex vivo* killing capacity [184] and restricted cytokine production [180]. Polyfunctionality of HIV-specific $CD8^+$ T-cells (perform multiple effector functions simultaneously, including the secretion of several cytokines and chemokines as well as degranulation) is associated with control of viral RNA-load [185]. Furthermore, patients without progressive infection exhibit increased frequencies of polyfunctional HIV-specific T-cells compared with those with disease progression [186]. A greater breadth of virus specific CTL by analysis of their T-cell receptor (TCR) repertoire has also been shown to be associated with better clinical status [187]. In addition, antigen sensitivity (functional avidity) of epitope-specific $CD8^+$ T-cells is associated with an effective HIV-specific T-cell response [186].

CTL determinant selection appears to be important in control of viral replication. A number of studies have found a positive association between responses targeted to Gag and viral control [188, 189]. One explanation which has been proposed for this is that changes in the Gag protein which facilitate viral escape may only occur to a certain extent before the associated change in structural or functional aspects of the protein leads to a compromise in replicative fitness [190].

Further evidence that CTLs have a significant role in ongoing containment of the virus comes from studies demonstrating that they select virus escape mutations as early as 30–54 days after the peak viral RNA-load of acute infection [83, 191] and continue to select mutations throughout chronic infection [192-194]. Correlation has been demonstrated between certain escape mutants and the prevalence of particular restricting class I HLA alleles in different cohorts [195].

Since viral escape from effective CTL is often associated with a cost in terms of replicative fitness [190], it could be predicted that specific class I HLA alleles would influence the rate of disease progression. This has indeed been shown to be the case as class I HLA alleles B57, B5801 and B27 are associated with slower progression to AIDS than individuals without these alleles [196-198].

The $CD4^+$ T-helper cell response is essential in maintaining effective $CD8^+$ T-cell responses to HIV-1. The 'help' provided by the $CD4^+$ T-cells enables CD8 T-cells to undergo secondary expansion upon re-stimulation [199]. They are activated when they bind a viral epitope presented on class II HLA molecules. Upon activation they secrete interleukin-2 (IL-2) and other cytokines that enhance the CTL and humoral responses [200]. Levels of these cells are likely to be affected since $CD4^+$ T-cells are the main target population for HIV-1. However, the fact that $CD4^+$ T-cell proliferative responses to p24 antigen (but not to cytomegalovirus antigen) in HIV-infected individuals were increased by administration of CD40 ligand and IL-12 (both agents which enhance responsiveness to antigen) suggests that qualitative differences, rather than merely depletion, may contribute to unresponsiveness in this compartment [201].

1.7.2 Host-derived Suppression of the CTL Response

As chronic infection ensues, HIV-specific CTLs typically begin to lose function [182, 184, 202]. This may be a result of persistent antigenic stimulation, leading to chronic immune activation which can lead to immune exhaustion [203]. Regulatory T-cells (T_{reg}) and T helper (Th) 17 cells are two recently described lymphocyte subsets with opposing actions which appear critical to regulation of the CTL response. T_{reg} regulate chronic inflammation by suppressing the activation and proliferation of effector lymphocytes and therefore maintain tolerance to self-antigens, but may also limit the vigor of the antimicrobial immune response [204]. T_{reg} are $CD4^+$ T-cells which constitutively express CD25 and CTLA-4, in addition to the transcription factor $Foxp3^+$ [205]. The effect of T_{reg} in HIV-1 infection is unclear since they may act to reduce activation (immune activation may drive HIV-1 pathogenesis, see Section 1.9.1) but may also limit HIV-specific adaptive responses [206]. Many studies report an expansion of this

population in the peripheral blood in HIV-1 and SIV infection [207, 208] and yet others find a decrease [209]. The differing methods used to identify these cells may account for these conflicting reports. Detection of T_{reg} in lymphoid tissue has been reported to be associated with viral RNA-load [209] and disease progression [210]. Further evidence for a role in chronic HIV-1 infection comes from the fact that depletion of $CD4^+CD25^+$ T-cells from peripheral blood mononuclear cells (PBMCs) from HIV-1 infected donors results in increased virus specific T-cell responses [211, 212]. Th17 cells appear to enhance host defences against infection and consequently appear to drive inflammation [213]. Further to this, it appears that it is an imbalance of T_{reg} cells and Th17 cells which drives the pathogenesis of chronic viral infections such as hepatitis B [214]. Progressive HIV-1 infection has been shown to be associated with a decreased proportion of memory Th17 cells and a reciprocal increase in T_{reg} cells [215].

Several studies have examined the role of programmed-death 1 (PD-1), an inhibitory molecule upregulated on T-cells during HIV-1 infection [216]. Studies have been conflicting in terms of whether PD-1 expression (and the associated decreased $CD8^+$ proliferative capacity seen) is an effect of high viral RNA-load [217] or whether its expression and associated T-cell dysfunction causes higher viral RNA-load [218]. Recent work suggests PD-1 is not merely a marker of viral RNA-load and exhaustion, since PD-1 blockade *in vitro* restores HIV-specific T helper responses [219].

1.7.3 Humoral Immunity

Following primary infection with HIV-1 and the subsequent development of specific $CD4^+$ T-cells a HIV-1 specific antibody response is detectable, although most of these antibodies are directed against virion debris [220]. Subsequently antibodies which bind to epitopes critical to viral entry are formed; these antibodies are termed 'neutralising antibodies' (nAb) due to their ability to block infection of susceptible cells [221]. For many years, it was thought that nAbs played little or no role in control of viral RNA-load during acute infection as these antibodies were found to be largely absent during this period [222-224]. Studies are likely to have been hampered by the fact that nAb responses to autologous viruses are difficult to measure because of the technical challenges associated with the preparation of autologous virus stocks. However, subsequent studies show that these antibodies can develop within weeks of infection and so may contribute to early control of viral RNA-load [225, 226].

Most often the nAb response is highly isolate-specific, i.e. efficient responses against heterologous isolates are rare. However, in some individuals the response matures and becomes

cross-neutralising, with prevention of infection by divergent strains (broadly nAbs) [227, 228]. nAbs are typically directed against functionally important receptor and co-receptor binding sites in the gp120 subunit, and antibodies against the ectodomain of gp41 subunit have been identified. In general, it appears that nAbs directed against gp120 are more potent but not as broad as nAbs against gp41 [229, 230].

Longitudinal studies show that HIV-1 rapidly escapes the nAb [226, 231, 232], which drives a continual evolution of envelope proteins. This escape is so rapid it is considered to be the reason many studies show weak or no neutralisation of autologous virus by contemporaneous Abs [225, 226].

The binding of antibody to the virion or infected cells may trigger downstream effector functions through its Fc portion. Effector functions include activating the complement cascade or opsonisation by binding Fc receptors and can both reduce and enhance virus replication through different mechanisms [233].

The HIV-1 virus employs a wide range of mechanisms by which it evades immune antibody recognition. The Env spikes on the virion surface are largely protected through occlusion of epitopes within the trimeric structure [234], steric and conformational blocking of the receptor binding sites [235] and extensive glycosylation [226, 236].

1.7.4 Innate Immunity

NK cells, a central component of the innate immune system, have been found to play a significant role in the control of HIV-1 replication [237-239]. This effect is found to occur as a result of cell-mediated cytotoxicity as well as noncytolytic suppression of viral replication through the production of chemokines [240]. In addition, HIV-binding antibodies allow NK cells to mediate antibody-dependent cellular cytotoxicity (ADCC), which probably also contributes to control of virus [241]. Given the central role of NK cells in HIV-1 control, it is not surprising the virus has developed mechanisms to avoid killing by these cells. For example, there is evidence for HIV-1 Vpu-mediated down modulation of NTB-A [242], which is important since NTB-A is a co-activating receptor necessary for NK cytolysis.

The result of the interplay between host immune response and viral replication during the acute phase is the establishment of a viral RNA-load set point a few months after infection. This is an important value since the higher it is the faster the rate of disease progression [243, 244].

1.8 Prevention of HIV-1 Infection

Education about routes of transmission remains central to the prevention of infection and curbing the epidemic. Numerous other interventions to prevent HIV-1 infection have also been studied. Mother-to-child transmission may be reduced from around 25% to as low as 1% by starting the mother on ART during pregnancy, Caesarian section, post-exposure prophylaxis with ART for the neonate when born and avoidance of breast-feeding [53]. A body of evidence has accumulated suggesting male circumcision may have a protective effect against HIV acquisition. This observation may be explained by the large number of HIV-susceptible dendritic cells in the foreskin [245]. Whilst the mechanism is not completely understood, it may be that this becomes part of effective HIV-prevention programmes [246].

Other prevention strategies which employ the use of ART include post-exposure prophylaxis following needle-stick accidents, where an 81% reduction in the risk of infection is seen [247]. Topical antiretrovirals such as tenofovir gel applied to the vaginal vault have been used to prevent transmission, with one study finding an overall 39% reduction in HIV-1 acquisition [248]. Trials using polyanionic microbicides have failed and may even have led to increased transmission [249]. The use of CCR5 antagonists as topical agents has a particularly strong rationale, and has been shown in rhesus macaques to protect against infection with CCR5-using virus [250]. Pre-exposure prophylaxis with oral ART has been studied in a large randomised double blind placebo controlled trial using once daily tenofovir with emtricitabine; a 75% reduction in HIV-1 transmission in serodiscordant heterosexual couples was observed [251].

It is widely believed the development of an effective vaccine is the most cost-effective and globally applicable public health approach to controlling the epidemic. The major difficulty in development is the marked heterogeneity of the virus [25]. Work in nonhuman primate models have demonstrated the protective effect of nAbs [252]. Furthermore, in humans the lack of nAb response to HIV-1 predisposes to superinfection [253].

Unfortunately, most vaccine trials have not demonstrated efficacy [254]. However, one trial using a prime/boost vaccine (RV144), which induces antibodies which bound but do not neutralise the virus, demonstrated a modest 31% reduction in the acquisition of infection [255]. No effect on post-infection viral RNA-load or $CD4^+$ T-cell count was seen in vaccinees who did acquire the virus. In addition, numerous T-cell vaccines have been developed which were hoped to protect from disease progression rather than from infection itself. As yet these have been unsuccessful, with one trial which used a recombinant adenoviral vector containing viral proteins reporting an increased likelihood of acquisition in those receiving the active vaccine

[256]. This finding highlights the extreme complexity of immune protection and response to infection, and the fact that understanding of these is not yet complete.

1.9 Mechanism of $CD4^+$ T-cell Depletion in HIV-1 Infection

The mechanisms leading to progressive decline of $CD4^+$ T-cells in HIV-1 infection are still not yet fully understood. The fact that higher viral RNA-load appears to predict $CD4^+$ T-cell decline and that HIV-1 is cytopathic for T-cells *in vitro* initially suggested that progressive decline of these cells *in vivo* may be due to the direct effects of ongoing infection of these cells (over and above supply of new cells from the thymus). However, subsequent studies have shown that during chronic HIV-1 infection more $CD4^+$ T-cells die than can be accounted for by direct infection [257] and an increased death rate is observed even in non-target cells, such as $CD8^+$ T-cells [258], suggesting an alternate mechanism. Furthermore, it appears that the extent of viral replication as measured by viral RNA-load may explain less than half of the variability in the rates of $CD4^+$ T-cell decline [259, 260]. Consequently, death of uninfected bystander cells via apoptosis has been proposed to play a major role in $CD4^+$ T-cell death [261].

1.9.1 Generalised Immune Activation in HIV-1 Infection

One process by which HIV-1 might lead to apoptosis of uninfected T-cells is as a result of immune activation [262]. Chronic HIV-1 infection is characterised by high levels of activation of both $CD4^+$ and $CD8^+$ T-cells [263]. Other studies which support this include that by Giorgi *et al*. which demonstrated that immune activation of $CD4^+$ and $CD8^+$ T-cells (upregulated CD38 expression) was strongly associated with shorter subsequent survival [264]. Sousa *et al.* demonstrated that in HIV-1 infection $CD4^+$ T-cell depletion was associated with higher levels of $CD4^+$ (upregulated HLA-DR and CD69 expression) and $CD8^+$ T-cell activation (upregulated HLA-DR and CD38 expression) [265]. This group also report similar immune activation levels in HIV-1 and HIV-2 for a given $CD4^+$ T-cell count, despite the much lower viral RNA-load in HIV-2 infection [265]. Other investigators have similarly reported a correlation between immune activation and disease progression in HIV-1 infection [263], with a stronger correlation of $CD4^+$ T-cell decline with immune activation than with viral RNA-load [266, 267].

Compelling evidence for the importance of T-cell activation in the pathogenicity of HIV-1 comes from the study of natural and non-natural SIV infection. As discussed in Section 1.1, wild primates in Africa frequently harbour natural SIV infection. SIV infection of Asian primates, such as rhesus macaques, is not seen in the wild [268]. Silvestri *et al.* showed that although SIV MM infected sooty mangabeys (i.e. the natural host of the virus) develop high

viral RNA-loads, they do not experience $CD4^+$ T-cell depletion or simian AIDS and increased T-cell activation is not seen ($CD4^+$ T-cell activation as demonstrated by HLA-DR expression, $CD8^+$ T-cell activation as demonstrated by CD69 expression). By contrast when rhesus macaques are infected with SIV MM (i.e. not the natural host of this virus) chronic activation is seen with progression to simian AIDS [269]. Other investigators have since published similar findings implicating immune activation in pathogenicity of non-natural SIV infection [270, 271]. The exact mechanisms underlying this phenomenon are still not completely understood, but it has been proposed that hosts of natural SIV down-regulate the immune responses to SIV via immune regulatory pathways [272].

The process leading to HIV-induced immune activation is not well understood. A potential mechanism which has been proposed is that HIV-1 Nef lowers the threshold required for T-cell activation via TCR stimulation [273]. Defective virions [274] and altered cytokine spectra [275] during HIV-1 infection have also been suggested as potential mechanisms leading to immune activation. It has also been suggested to result from breakdown of the mucosal barrier due to the massive depletion of $CD4^+$ T-cells at this site. This may lead to the translocation of microbial products, such as bacterial lipo-polysaccharide (LPS), from the intestinal lumen to the systemic circulation, causing generalised immune activation [276].

It is clear that immune activation is likely to be beneficial for HIV/SIV since the virus replicates far more efficiently in activated $CD4^+$ T-cells compared with resting $CD4^+$ T-cells [111, 277]. Furthermore, bystander activation of uninfected $CD4^+$ T-cells increases susceptibility to infection and subsequent virus production *in vivo* [112]. In this way it is likely the virus maintains the supply of target activated $CD4^+$ T-cells despite overall declining numbers of $CD4^+$ T-cells [112].

1.9.2 Specific Targeting of CD4$^+$ T-cells by HIV-1

Although there is significant evidence implicating immune activation in the mechanisms leading to CD4$^+$ T-cell destruction, it is not fully understood why there is selective depletion of CD4$^+$ T-cells when HIV-1 activates both CD4$^+$ and CD8$^+$ T-cells [265]. One proposed mechanism which may account for the specific destruction of CD4$^+$ T-cells involves interferon (IFN)-α, a cytokine commonly produced directly in response to viral infection. HIV-1 gp120 induces monocyte and dendritic cell IFN-α production *in vitro* [278]. These *in vitro* models demonstrate that IFN-α may drive CD4$^+$ T-cell apoptosis and death by upregulating TNF-α related apoptosis inducing ligand (TRAIL) expression in CD4$^+$ cells [278, 279]. However, this proposal is far from clear since IFN-α has been shown to inhibit HIV-1 replication *in vitro* [280, 281] and *in vivo*, albeit to a modest extent [282, 283]. In addition, there is evidence suggesting IFN-α may protect T-cells from apoptosis [284], highlighting further the complexity of its action in this setting.

Another mechanism which might account for apoptosis of uninfected CD4$^+$ T-cells results from interaction with the HIV-1 surface Env. Env has long been suspected to be a likely candidate for inducing bystander cell death, since it is expressed on the surface of infected cells and is therefore readily available for interaction with nearby uninfected cells. As it binds CD4 and co-receptors, this might account for specific depletion of CD4 expressing T-cells [285]. Env may lead to bystander death through gp41-mediated hemifusion. Hemifusion is a process whereby the outer membrane of the Env-expressing infected cell and bystander cells interact transiently without fusion causing membrane destabilisation and apoptosis in the uninfected cell [286]. In keeping with this, an *in vitro* study showed that a mutation in the fusion domain of gp41 results in a fusion-defective Env unable to induce apoptosis [286]. Whatever the balance of these alternate mechanisms, it is likely that the accelerated apoptosis of CD4$^+$ T-cells in HIV-1 infection is multifactorial [287].

A further process by which Env may result in specific depletion of uninfected CD4$^+$ T-cells in HIV-1 infection is NK cell-mediated killing of these cells. This is thought to be a result of recognition of cellular ligand of NKp44 on CD4$^+$ T-cells [288]. NKp44L expression on CD4$^+$ T-cells is thought to be induced by a portion of HIV-1 gp41 [288].

1.9.3 Sequestration or Impaired Production of $CD4^+$ T-cells in HIV-1 Infection

Sequestration of $CD4^+$ T-cells to secondary lymphoid tissues or other sites is another possible mechanism leading to apparent depletion of circulating $CD4^+$ T-cells. Circulating $CD4^+$ T-cells represent only 1–2% of the body's total reservoir, with large proportions in muscoal sites and secondary lymphoid tissue [289]. Enhanced sequestration to secondary lymphoid tissues during chronic HIV-1 infection is supported by the observation that ART leads to increased circulating $CD4^+$ T-cells due to redistribution from these sites [290]. The ability of lymphocytes to migrate to specific areas of the body, such as the skin or the gut, is mediated in part by cell-surface molecules that tether cells to molecular partners expressed on the vascular endothelium at those sites [291]. For example, migration of lymphocytes to GI tract is mediated by integrin $\alpha 4\beta_7$ [292].

The reduction in $CD4^+$ T-cell count over time also appears to result, at least in part, from impaired production of T-cells. Evidence of suppression of multi-lineage haematopoiesis has been demonstrated in HIV-1 infection [293, 294]. Furthermore, HIV-1 infection has been shown to lead to a decline of both $CD4^+$ and $CD8^+$ naive T-cells in the peripheral blood as disease progresses [265, 295, 296] which may reflect decreased thymic production.

These mechanisms are not mutually exclusive. For example, the thymus (which may be defective to some degree) may replenish the increased loss of T-cells resulting from chronic activation for some time until a threshold is reached and supply cannot meet demand.

1.10 HIV-1 Viraemic Controllers

Patients who remain healthy or achieve viral control in the absence of ART are crucial to understanding of HIV-1 pathogenesis. Initial work focused on so-called long-term nonprogressors (LTNPs) who are HIV-1 infected and remain asymptomatic with $CD4^+$ T-cell count >500 cells/mm^3 for more than 10 years without treatment. Most but not all of these patients have low viral RNA-loads [297]. Attention shifted somewhat since then to those patients who maintain low viral RNA-load without treatment, termed viraemic controllers (typically defined as <2000 copies/ml but definitions vary between investigators). A rare subgroup of these controllers are termed 'elite-controllers' since viral RNA-loads are consistently below the limit of detection of current standard assays (typically 40 - 50 copies/ml) [298].

The mechanism by which this apparent control occurs is not fully characterised. Studies on elite controllers using co-culture assays and full genome sequencing of virus have revealed replication–competent virus without any gross defects [299]. More recent studies in which chimeric viruses containing gene segments from virus from elite controllers have shown that changes in *gag* and *pol* genes from these patients contribute to a virus which has low replicative fitness [300]. Whether this reduced replicative fitness is due to escape from effective CTL response, or elite controllers are infected with a virus with impaired replicative capacity, or a combination of these factors, is the matter of ongoing debate [190, 301].

The fact that it has been observed that an elite controller maintained control when superinfected with a second HIV-1 isolate would argue that the host immune response is most important [302]. Further support for this comes from the observation that virus has been transmitted from a patient who subsequently progressed to AIDS to an individual who became an elite controller demonstrating control of the fully pathogenic HIV-1 isolate [303].

The critical importance of the cell-mediated immune response in contributing to control in viraemic controllers has been well documented. For example, the protective class I HLA alleles B57 and B27 discussed in Section 1.7.1 are over-represented amongst viraemic controllers [304]. Further sites in the class I HLA-C allele have also been identified as being associated with viral control in this cohort using whole-genome association analysis [305]. $CD8^+$ T-cell polyfunctionality, as measured by secretion of multiple cytokines, has been shown to be stronger in elite controllers than in those with ART-mediated viral suppression [185]. Interestingly, nonhuman primates achieving elite control of SIV (SIVmac239 infected macaques), in which there is an over-representation of class I HLA alleles *Mamu*-B08 or B17,

bind similar peptides to HLA B57 and HLA B27 [306]. This suggests that determinant selection is important in control of virus [306]. In line with this, it has been demonstrated that viraemic controllers show preferential $CD8^+$ T-cell targeting of the HIV-1 Gag protein [304]. A further study has also shown selection of rare Gag mutants in HLA B57/B5801 elite controllers (compared with viraemic individuals) which have decreased replicative capacity and are a result of strong CTL responses [307]. Given the key role $CD8^+$ T-cells play in maintaining viral control, it is likely that $CD4^+$ T-cell help is also important. HIV-specific $CD4^+$ T-cells have been shown to demonstrate higher functional avidity in elite controllers than in patients with high viral RNA-load [200].

Since the GI tract has been shown to be a site of persistent HIV-1 replication and $CD4^+$ T-cell depletion [308], $CD4^+$ T-cells populations and mucosal $CD8^+$ T-cell responses have been studied in elite controllers, viraemic controllers, patients with uncontrolled viraemia ('non controllers', here defined as viral RNA-load >10000 copies/ml) and individuals on ART [309]. Mucosal $CD4^+$ T-cells were found to be significantly higher in elite and viraemic controllers compared with those in non controllers suggesting preservation of this compartment. Mucosal $CD8^+$ T-cell responses in controllers (elite and viraemic together) were found to be significantly stronger and more complex than in those patients on ART. In addition, there was an increased frequency of rectal polyfunctional $CD8^+$ T-cell response in controllers compared with non controllers and individuals on ART [309]. This suggests that $CD8^+$ T-cell responses in the gut mucosa play an important role in viral control in elite and viraemic controller cohorts.

In keeping with observations described in progressive HIV-1 disease, elite controllers have been shown to have lower levels of T_{reg} cells compared with non controllers (here defined as viral RNA-load > 4000 copies/ml) [310]. This group also demonstrated a Th17/ T_{reg} ratio that was similar in elite controllers compared with uninfected individuals and that this was lower than in non controllers and patients on ART [310]. Thus it appears that control of viraemia may be dependent on maintaining a normal balance between Th17 and T_{reg}.

The fact that nAbs select for HIV-1 escape mutants would suggest that these antibodies provide a significant contribution to viral control during chronic infection [226, 231, 232]. However, studies suggest that elite controllers do not harbour broadly nAbs as frequently as non controllers [228, 311]. It was therefore investigated whether nAbs in elite controllers might be directed specifically to autologous virus rather than a broad range. A study by Bailey *et al.* showed that this was not the case since elite controllers had similarly low levels of nAbs to autologous, contemporaneous plasma virus compared with progressors [312]. Low levels of nAbs in elite controllers may result from lack of antigenic stimulation, as suggested by low

plasma viral RNA-load. Based on these observations it is currently unclear what contribution nAbs make to the elite or viraemic controller phenotype.

Since HIV-1 DNA-load is associated with disease progression [313], this would predict that elite and viraemic controllers would have lower levels since rates of progression are slower in these groups. In keeping with this Lambotte *et al.* studied 15 viraemic controllers from a cohort in France and found very low and stable HIV-1 DNA-loads in PBMC in all patients [314].

It is likely that control of viral RNA-load is multifactorial; the specific complexities of that control are the subject of much ongoing research.

Whilst low HIV-1 RNA-loads tend to be associated with more stable $CD4^+$ T-cell counts [243, 315] this is not the case for all patients. Madec *et al.* identified 145 viraemic controllers (viral RNA-load <500 copies/ml) and found five experienced $CD4^+$ T-cell decline [316]. Pereyra *et al.* examined 90 elite controllers (<50 copies/ml) and found that six patients had a median $CD4^+$ T-cell counts of <500 cells/mm^3 and two patients had a median of <300 cells/mm^3 [317]. Okulicz *et al.* compared $CD4^+$ T-cell counts in 25 elite controllers with those in 153 viraemic controllers (viral RNA-load <2000 copies/ml). Elite controllers experienced prolonged stability of their $CD4^+$ T-cell count, whereas viraemic controllers demonstrated gradual decrease in $CD4^+$ T-cell over time [318]. Boufassa *et al.* studied a group of 106 viraemic controllers (unconventional definition of viral RNA-load <400 copies/ml) and found that $CD4^+$ T-cell counts fell in subgroups which experienced transient increases in viral RNA-load [319].

1.11 The Aims of this Thesis

Prior to starting the work described in this thesis, a notable number of patients were being identified in the HIV clinics in East London with control of viraemia (viraemic controllers) and yet low $CD4^+$ T-cell counts. At that time only one of the papers described in the last section on this patient group was published [316].

For this reason work described in this thesis was designed to define this population of viraemic controllers with low $CD4^+$ T-cell counts, referred to here as 'discord controllers', and to determine what features might distinguish them from 'typical controllers', those viraemic controllers with normal $CD4^+$ T-cell counts.

The specific aims of the work submitted were to:

- Determine whether there are epidemiological or virological factors that distinguish discord controllers from typical controllers (Chapter 2).
- Establish whether ART in discord controllers results in recovery of $CD4^+$ T-cell counts (Chapter 2).
- Determine whether changes in the T-cell compartment typically associated with HIV-1 disease progression (naïve T-cell depletion and T-cell activation) are more marked in discord controllers compared with typical controllers (Chapter 3).
- Determine whether HIV-1 DNA-loads are higher in discord controllers compared with typical controllers (Chapter 4).
- Determine whether HIV-1 DNA-load in viraemic controllers predicts subsequent HIV-1 RNA-load peak values or change in $CD4^+$ T-cell counts (Chapter 5).
- Investigate whether there are higher levels of migration of $CD4^+$ T-cells to the GI tract in discord controllers (Chapter 5).

CHAPTER 2

Epidemiological and Virological Characteristics of Discord Controllers and Typical Controllers, and Response to Antiretroviral Treatment of Discord Controllers

2.1 Introduction

Viraemic controllers are rare patients with the capacity to control viral RNA-load in the absence of ART [298]. The reasons why control of viral RNA-load is possible in these patients is incompletely understood. This cohort is composed of both viraemic controllers defined as those patients whose viral RNA-loads are below 2000 copies/ml and elite controllers in whom viral RNA-load is consistently below the limit of detection of current standard assays (typically 40 - 50 copies/ml) [298, 304]. Whilst these patients usually maintain normal $CD4^+$ T-cell counts, a subset have low or declining $CD4^+$ T-cells counts as described previously [320, 321]; these patients are referred to here as 'discord controllers'.

The first aim of work described in this Chapter was to determine whether there are epidemiological features or viral factors which distinguish discord controllers (low $CD4^+$ T-cell count) from typical controllers (normal $CD4^+$ T-cell count). To do this a local cohort of viraemic controllers was identified, with identification of the subset within them with low $CD4^+$ T-cell counts (<450 cells/mm^3); this group was then compared with those patients with normal $CD4^+$ T-cell counts (>450 cells/mm^3). Whilst other groups have recorded age, sex and ethnicity of viraemic and elite controllers [304, 318], these groups did not examine how epidemiological characteristics were represented in viraemic controllers with normal $CD4^+$ T-cell counts compared with those with low $CD4^+$ T-cell counts as is presented here.

The second aim of work described in this Chapter was to determine $CD4^+$ T-cell count change over time in discord controllers started on ART to further determine whether their low $CD4^+$ T-cell counts were normal for the particular patient or a result of HIV-1 driven depletion; this latter possibility would be supported by $CD4^+$ T-cell gains whilst on ART. It was also considered of clinical interest to record whether $CD4^+$ T-cell counts recover in the same way that they do in non controllers started on ART. This was reported on during the course of this work by another group [322]. Findings presented here are compared and contrasted with findings by that group.

2.2 Methods

The research protocol for work described in this and subsequent Chapters was approved by East London and The City Local Research Ethics Committee (No. 06/Q0603/59).

2.2.1 Study Groups

Patients were recruited, after obtaining informed consent, from outpatient clinics at Barts and The London NHS Trust and Homerton University Hospital Trust. All patients were shown to be HIV-1 seropositive and HIV-2 seronegative with the exception of one patient. For this patient, on whom serology was not available, sequence analysis of virus from blood demonstrated HIV-1 infection. Elite controllers were defined as those with undetectable viral RNA-load, although occasional non-consecutive blips of viral RNA-load up to 1000 copies/ml were permitted. Viraemic controllers were defined as those viraemic controllers who did not fit within the elite controller definition but have viral RNA-load below 2000 copies/ml. Both definitions are based on at least three viral RNA-load measurements over at least 12 months without ART. Patients were permitted to have been treated with ART previously (e.g. during pregnancy), as long as control based on three viral RNA-load over at least 12 months could be demonstrated. These definitions are in line with those used by the International HIV Controller Consortium [298]. The viraemic controller cohort as a whole was divided into two groups based on the geometric mean of the last three $CD4^+$ T-cell counts: typical controllers with >450 cells/mm^3 and discord controllers with <450 cells cells/mm^3.

Epidemiological data was recorded including age, sex, ethnicity, country of birth, and blood borne virus risk behaviour. Date of first positive HIV-1 test was also recorded. Co-morbid and intercurrent illnesses during follow-up were recorded for each patient.

Patients followed up in the clinic were seen at 4 to 12 monthly intervals and had blood taken for routine laboratory evaluation including $CD4^+$ T-cell count and viral RNA-load.

2.2.2 Routine HIV-1 RNA-load and CD4$^+$ T-cell Measurements

For patients recruited from Barts and the London NHS Trust, routine laboratory CD4$^+$ T-cell counts and percentages were analysed by Barts and the London NHS Trust Department of Immunology. The blood samples were processed with the BD FACS Sample Prep Assistant with Trucount beads and were then acquired on a BD FACSCanto II flow-cytometer. FACSCanto clinical software was used for data analysis. Samples from patients recruited from Homerton University NHS Trust were processed by 'The Doctors Laboratory' using the Beckman Coulter Navios Flow cytometer.

HIV-1 RNA-load was measured using Roche Ampliprep/COBAS Taqman HIV-1 Test v1.0 (Roche Molecular Systems, Inc., Blanchburg, New Jersey; detection limit of 40 copies/ml). At least one sample from each patient was also tested using a different assay to confirm the low viral RNA-load and thus ensure that the initial result was not simply due to underquantification by a single platform. These assays were carried out by Barts and the London NHS Trust Diagnostic Virology Laboratory.

Individual patient viral RNA-load and CD4$^+$ T-cell counts are reported as the geometric mean of the last three measurements. In addition to determining mean absolute CD4$^+$ T-cell counts in each controller cohort, mean CD4$^+$ T-cell percentage was calculated. This was done by calculating the mean of the last three CD4$^+$ T-cell percentage values. If a patient had achieved viraemic control (viral RNA-load <2000 copies/ml) but subsequently viral RNA-load rose above this, the last date of viraemic control was determined to be the last date a viral RNA-load measurement would still contribute to a geometric mean of <2000 copies/ml. The three viral RNA-load and CD4$^+$ T-cell values prior to this date were used to calculate the mean for that patient. Similarly, for patients who started ART during follow-up the three viral RNA-load and CD4$^+$ T-cell values prior to starting ART were used to calculate the mean.

Those discord controllers who were started on ART were followed: viral RNA-load, absolute CD4$^+$ T-cell count and CD4$^+$ T-cell percentage were recorded.

2.2.3 HIV-1 Subtype Analysis

Some HIV-1 subtype data was available as part of the anti-retroviral resistance testing (sequence obtained from *pol*: whole of protease and amino acid 1-335 of reverse transcriptase). This was carried out by Barts and the London NHS Trust Diagnostic Virology Laboratory. The assay has demonstrated efficacy in identifying a wide variety of subtypes including recombinant subtypes [67]. Other papers have demonstrated subtype determination (including recombinant subtypes) using assays which amplify this region of *pol* [323, 324]. This laboratory provides HIV-1 anti-retroviral testing widely for North-East and East London; the population of patients for which the laboratory provides HIV-1 assays will be referred to from here as the East London HIV-1 population.

For patients on whom anti-retroviral resistance testing was unavailable, previously described *env* [325] and *gag* [323] nested PCRs were used in parallel to accurately determine recombinant subtypes. DNA for this was extracted from cryopreserved PBMCs, prepared by Ficoll-Hypaque separation of ethylenediaminetetraacetic acid (EDTA) anticoagulated blood (see Appendix I), using the Qiagen EZ1 biorobot and DNA Tissue programme according to the manufacturer's instructions into a 200µl eluate.

For the *env* PCR a final 25µl PCR mixture containing 5µl of the DNA extracted from PBMC, 10× Hotstar PCR buffer (Qiagen, containing 15mM $MgCl_2$), additional 0.5mM MgCl, 0.2mM dNTPs, 0.5µM of each primer (F1 5'-TCT TAG GAG CAG CAG GAA GCA CTA TGG G-3'; R1 5'-AAC GAC AAA GGT GAG TAT CCC TGC CTA A-3') and 1.25 units of Taq. Thermocycling conditions; 95°C for 15 minutes; 40 cycles of 95°C for 30 seconds, 50°C for 30 seconds, 72°C for 1 minute; and 72°C for 5 minutes. 1 µl of the first-round PCR product taken for second-round PCR in which the final concentration of reagents were the same as for the first except the primers which were used at a final concentration of 0.25µM (F2 5'-ACA ATT ATT GTC TGG TAT AGT GCA ACA GCA-3'; 5'- TCC TAC TAT CAT TAT GAA TAT TTT TAT ATA-3'). PCR cycling conditions were as the first round and produced a 445 kilobase fragment. For the *gag* PCR a final 25µl PCR mixture containing 5µl of PBMC DNA, 10× Hotstar PCR buffer, additional 1mM MgCl, 0.2mM dNTPs, 0.5µM of each primer (F1 5'–TCACCTAGAACTTTGAATGCATGGG-3'; R1 5'–CTAATACTGTATCATCTGCTCCTGT-3') and 1.25 units of Taq. Thermocycling conditions; 95°C for 15 minutes; 40 cycles of 95°C for 30 seconds, 48°C for 30 seconds, 68°C for 1.5 minutes; and 68°C for 5 minutes. 1 µl of the first-round PCR product was used in each second-round PCR in which final concentration of reagents was the same as for the first round (F2 5'-AAAGATGGATAATCCTGGG-3'; 5'-TCCACATTTCCAACAGCCCTTTTT-3'). Cycling conditions were 95°C for 15 minutes; 40

cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute; and 72°C for 5 minutes, producing a 470 kilobase fragment. Amplified products were detected on a 2% agarose gel and visualised using ethidium bromide. The PCR product was cleaned using Qiagen QIAquick PCR purification kit and sequenced using the ABI BigDye Terminator V1.1, Perkin Elmer, Foster City, CA and an automated sequencer (ABI 3100 Genetic Analyser, used according to the manufacturer's instructions). Both strands of the PCR products were sequenced directly using the second round forward and reverse primers and then cleaned for processing using the Sodium Acetate Ethanol protocol advised by ABI. The sequences were analysed using SeqScape V2.11 software. Manual corrections were made to ensure that gaps did not alter the reading frame. These were submitted to the REGA online typing tool (Stanford University, 2006) for genotype allocation, thereby allowing subtype prediction. All nested PCR work and sequencing reactions were carried out as part of the work presented in this thesis. Sequences generated were analysed by Dr David Bibby, in the Barts and the London NHS Trust Diagnostic Virology Laboratory.

2.2.4 Statistical Analysis

Statistical analysis and graphical presentations were performed using Prism (version 4.0, Graphpad) and results were considered significant if $p<0.05$. To determine significant differences in age, time since first positive test between each cohort, a Mann-Whitney U test (2-tailed) was applied. Fisher's exact test (2-tailed) was used when comparing sex distribution, ethnicity, country of birth, risk behaviour and subtype.

Due to the rarity of viraemic controllers even in a large total HIV-1 infected cohort, power calculations were not undertaken to determine sample sizes; instead, all patients fulfilling the controller inclusion criteria and available to consent to the study were included. Because small sample sizes increase the likelihood of Type II error, a lack of statistical significance in comparison between groups should be interpreted with caution.

2.3 Results

2.3.1 HIV-1 RNA-load and CD4$^+$ T-cell Values for Discord Controllers and Typical Controllers

A cohort of 82 viraemic controllers was established: 64 typical controllers and 18 discord controllers. Approximately 3000 patients attend the clinics, thus controllers represent 2.7% (discord controllers 0.6%, typical controllers 2.1%) of all infected patients. Figure 2.1 shows viraemic controllers with low viral RNA-load (<2000 copies/ml over at least 1 year), divided into typical controllers and discord controllers based on CD4$^+$ T-cells count, together with non controllers for comparison.

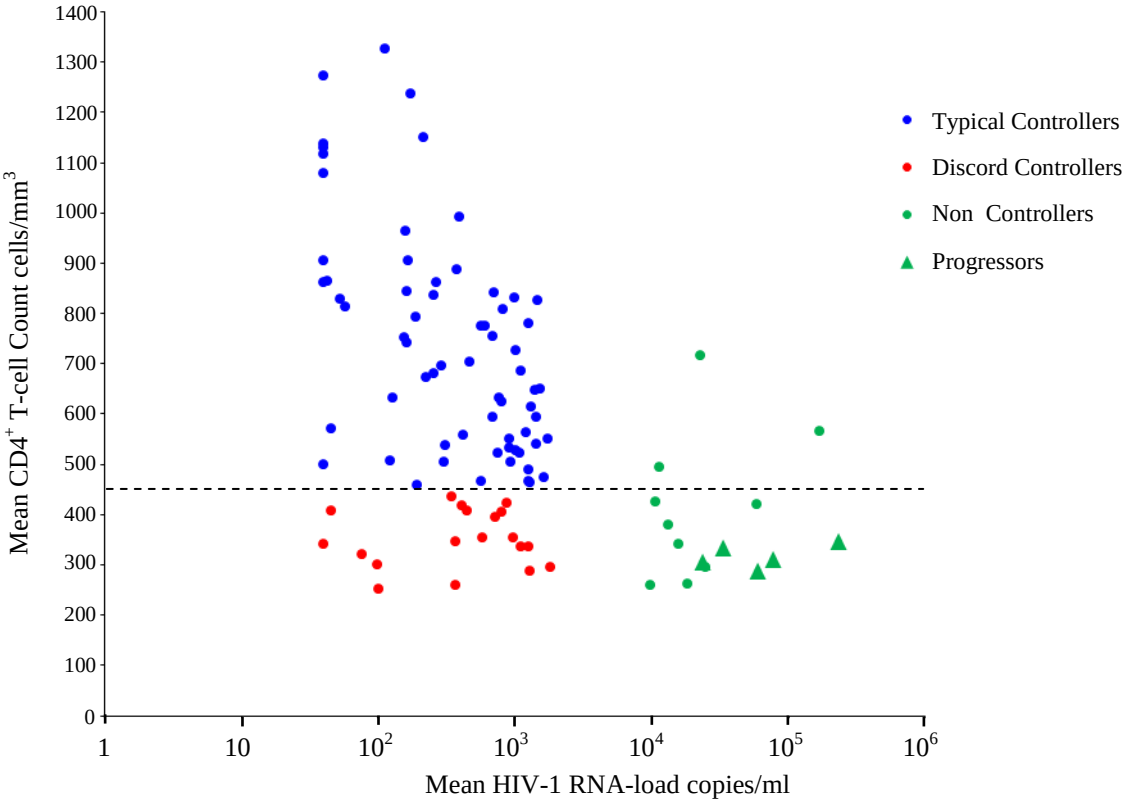


Figure 2.1 Mean Plasma HIV-1 RNA-load (copies/ml) and CD4$^+$ T-cell Counts in Patients in HIV-1 Positive Cohorts. Non controllers shown in green circles with a range of CD4$^+$ T-cell counts are used as controls for HIV-1 DNA load data (Chapter 4), and progressors (green triangles, a subgroup of non controllers with low CD4$^+$ T-cell counts) are a control group for T-cell data (Chapter 3). Mean CD4$^+$ T-cell count (cells/mm^3) and mean HIV-1 RNA-load (copies/ml) were calculated by determining the geometric mean of the most recent three values for each patient.

Mean viral RNA-loads in discord controllers and typical controllers were all below 2000 copies/ml, but were compared further in order to determine whether levels were equivalent below this threshold. Viral RNA-loads were found to be indistinguishable (p=0.144, see Figure 4.2 b). Most patients in both cohorts had detectable viral RNA-loads below the 2000 copies/ml cut-off. However, a few patients were found to be elite controllers (viral RNA-load < 40 copies/ml, occasional non-consecutive blips): two (11.1%) of the discord controllers could be classed as elite controllers, and 10 (15.6%) of the typical controllers. There was no significant difference of frequency of elite controller in each cohort using Fisher's exact test.

Viral RNA-loads remained stable for the majority of viraemic controllers; however, for a minority control was lost during follow-up. Careful assessment of the pattern of viral control prior to the point at which it was lost was important in order to determine to which cohort the controller belonged. For a minority of patients, classification was complicated by the fact that on occasion viral RNA-load was not requested during a clinic visit despite blood being tested for $CD4^+$ T-cell count. These groups of patients are described more fully in Appendix II.

Typical controllers had normal $CD4^+$ T-cell counts (median 699 cells/mm^3, interquartile range [IQR] 550-843) similar to findings in viraemic controllers cohorts [304]. The discord controllers had low $CD4^+$ T-cell counts (median 347 cells/mm^3, IQR 298-407).

Thirteen patients (3 discord controllers and 10 typical controllers) had been previously treated with ART during pregnancy, but had demonstrable control of viral RNA-load over at least 12 months subsequently.

$CD4^+$ T-cell percentage was also recorded in discord controllers and compared with typical controllers in order to help determine whether low $CD4^+$ T-cell counts were a result of specific depletion of this cell type or due to a lower numbers of all T-cell subsets which is seen in some ethnic groups. The geometric mean of the three most recent $CD4^+$ T-cell percentages for each patient in each cohort was calculated and results displayed in Figure 2.2.

Data presented in Figure 2.2 demonstrate that discord controllers not only have lower absolute $CD4^+$ T-cell count, they also have significantly lower $CD4^+$ T-cell percentage ($p < 0.0001$), suggesting a specific depletion of $CD4^+$ T-cells.

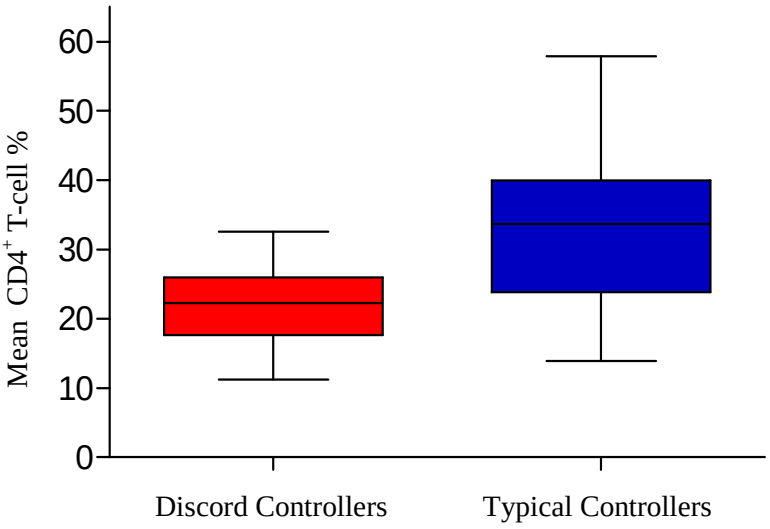


Figure 2.2 Mean $CD4^+$ T-cell Percentage for Discord Controllers and Typical Controllers. Horizontal line depicts median of these values, the box represents the IQR and the whiskers the range of values. Median (IQR): typical controllers: 33.7% (23.8 – 40.0), discord controllers: 22.3% (17.6 – 26.0).

2.3.2 Epidemiological and Clinical Features of Discord Controllers and Typical Controllers

Epidemiological characteristics were recorded for the discord controllers and typical controller cohorts in order to determine whether there were features that could distinguish discord controllers from typical controllers. Data as displayed in Table 2.1 shows there was no difference in median age (p=0.44), sex distribution (p=0.79), ethnicity, region of birth or risk behaviour. Co-morbid and intercurrent illness for each patient, including those which may be related to immunosuppression due to HIV infection, are recorded in Appendix III .

There was no significant difference comparing time since first positive test (best available surrogate for time since seroconversion) between the two controller cohorts (p=0.25). This, together with the similar age distribution, suggests that it is likely that the discord controller patients did not have a longer duration of HIV-1 infection than typical controllers.

Table 2.1 Epidemiological Characteristics of Discord Controllers and Typical Controllers

		Typical Controllers ($CD4^+$ T-cell count >450 cells/mm^3) *n* = 64	Discord Controllers ($CD4^+$ T-cell count <450 cells/mm^3) *n* = 18
Age, years		37.5 (32.0 - 43.0)	38.5 (32.5 - 49.0)
Female sex		35 (54.7%)	11 (61.1%)
$CD4^+$ T-cell count, cells/mm^3 [a]		699.3 (550.4 - 843.1)	347.2 (298.0 - 406.6)
HIV-1 RNA-load, log_{10} copies/ml [a]		408.5 (160.7 - 1000.0)	428.6 (100.3 - 1043.0)
Birth Place	Europe	25 (39.1%)	7 (38.9%)
	Africa (Sub-Sahara)	28 (43.7%)	10 (55.6%)
	Caribbean	6 (9.4%)	0 (0%)
	South Asia	3 (4.7%)	0 (0%)
	Other	2 (3.1%) (Australia. Ecuador)	1 (5.5%) (New Zealand)
Ethnicity	White British	12 (18.8%)	3 (16.7%)
	White other	8 (12.5%)	2 (11.1%)
	Black African (Sub Saharan)	28 (43.7%)	11 (61.1%)
	Black Caribbean	11 (17.2%)	2 (11.1%)
	South Asian	3 (4.7%)	0 (0%)
	Other	2 (3.1%) (Mixed White and Black African; South American)	0 (0%)
Time since first positive test, years		4.5 (2.5 - 6.5)	4.6 (3.3 - 9.3)
Risk behaviour	Heterosexual	42 (65.6%)	11 (61.1%)
	[Specified abroad]	[26 (40.6%)]	[5 (27.8%)]
	Homosexual	20 (31.3%)	6 (33.3%)
	Blood Products	1 (1.6%)	1 (5.6%)
	IVDU	1 (1.6%)	0 (0%)

Data presented in this table are median (IQR) or *n* (%). [a]median (IQR) of geometric mean calculated for each subject using the three most recent $CD4^+$ T-cell counts and HIV-1 RNA-loads.

2.3.3 HIV-1 Subtypes in Discord Controllers and Typical Controllers

Analysis of HIV-1 subtype in viraemic controllers was undertaken in order to determine whether discord controllers were different from typical controllers based on the frequency of subtypes isolated. This was compared with the subtype distribution in the East London HIV-1 population based on assays over a five year period (2004-9, 3010 patients, data was adjusted to remove patients with repeat testing).

For those patients on whom anti-retroviral resistance testing was not available, five discord controllers and 13 typical controllers, a nested PCR was used to amplify regions in *env* and *gag* for sequencing and subtype prediction (Table 2.2). Apart from two samples (mean viral RNA-load of <40 and 111 copies/ml; HIV-1 DNA loads of 47.8 and 0 copies/10^6 $CD4^+$ T-cells, respectively; see Figure 4.2), virus was successfully amplified and sequenced from all samples tested using the nested PCRs. For four patients anti-retroviral testing was carried out after subtype prediction was obtained by *env/gag* nested PCRs. Subtype prediction was found to be the same for four patients on whom both methods were used (one subtype C, one subtype B, and two subtype CRF02_AG).

The subtype distribution in the controller cohorts is shown in Table 2.2, and is also displayed and compared with the total East London HIV-1 infected population in Figure 2.3.

There was a non-significant trend towards an increased frequency of subtype C in the discord controllers compared with typical controllers and the East London HIV-1 population. In addition, there was a non-significant trend towards an increased frequency of subtype CRF02_AG in all viraemic controllers compared with the East London HIV-1 population.

Table 2.2 HIV-1 Subtypes in Discord Controllers and Typical Controllers

HIV-1 subtype	Typical Controllers ($CD4^+$ T-cell count > 450 cells/mm^3) n = 41	Discord Controllers ($CD4^+$ T-cell count < 450 cells/mm^3) n =15
B	14 (34.1%)	6 (40.0%)
C	7 (17.1%)	6 (40.0%)
CRF02_AG	8 (19.5%)	3[a] (20.0%)
A	4 (9.7%)	0 (0%)
F	2 (4.9%)	0 (0%)
CRF14_BG	2 (4.9%)	0 (0%)
Other	1 each of: D; A/C; A/D; B/D	0 (0%)

[a]for one sample, sequence analysis of nested PCR products indicated either CRF02_AG or CRF09_cpx.

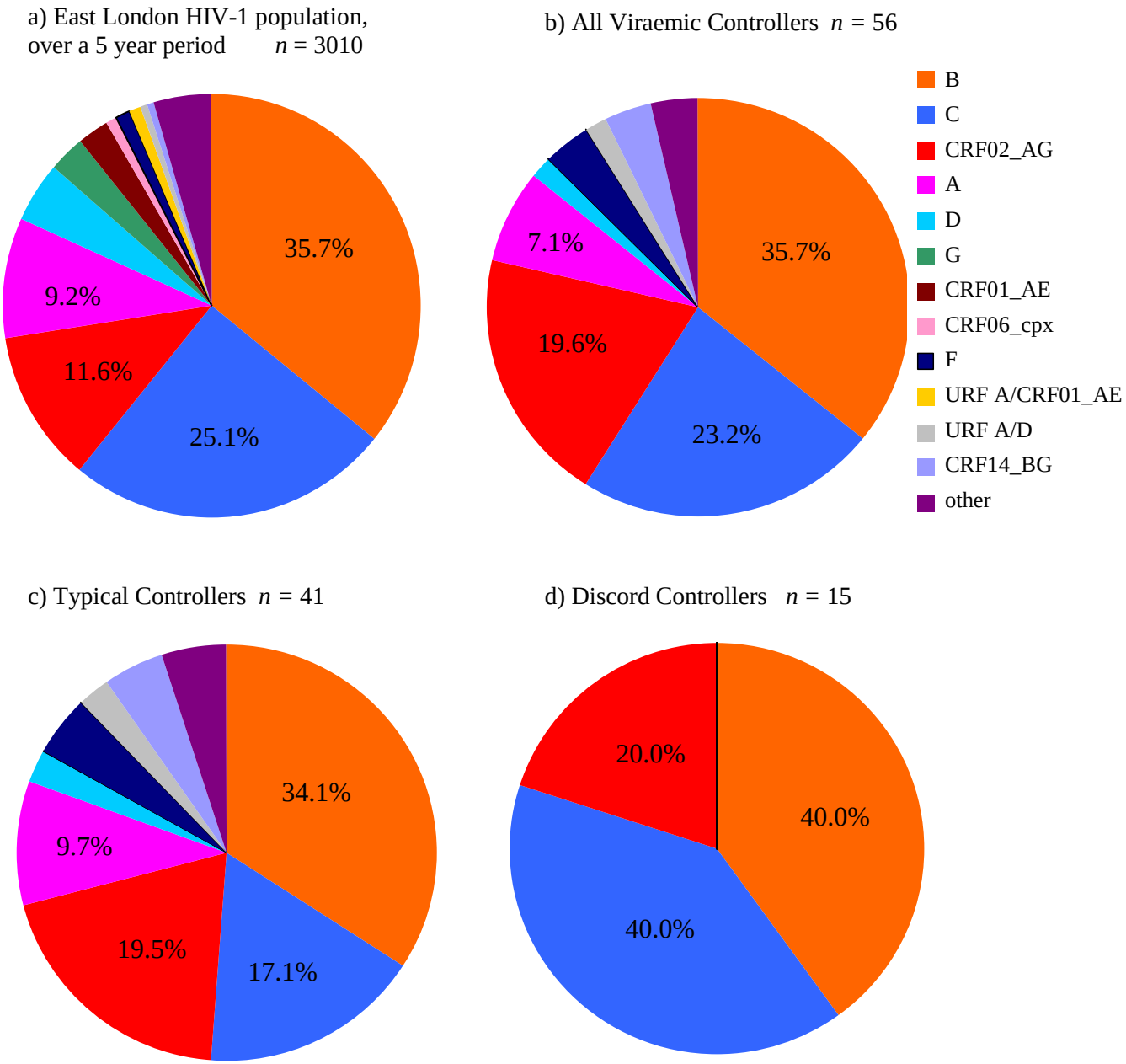


Figure 2.3 HIV-1 subtypes in Discord Controllers, Typical Controllers and the East London HIV-1 population. Percentages displayed for the East London HIV-1 population in (a) is for those patient in whom HIV-1 subtype was available as part of anti-retroviral resistance assay.

2.3.4 Response of Discord Controllers to ART

A number of patients in the discord controller cohort were started on ART during the course of this study. None of these patients had AIDS at the point of starting ART (i.e. $CD4^+$ T-cell count was not below 200 cells/mm^3 for any of these patients, and neither did they have AIDS defining conditions). Follow-up of these patients was of relevance in order to further determine whether their low $CD4^+$ T-cell counts were suboptimal as would be suggested by $CD4^+$ T-cell gains whilst on ART. It was also of clear clinical interest to clinicians to record whether $CD4^+$ T-cell counts recover in the same way that they do in non controllers started on ART. Patient 2 was the only elite controller amongst these patients started on ART. For the remaining patients, viral RNA-load was quickly suppressed to undetectable levels.

Figure 2.4 demonstrates $CD4^+$ T-cell counts where available over 24 months following start of ART for these patients (follow-up extended past this for Patient 4, see Table 2.3).

It can be seen that several of these patients responded to ART treatment with a slow and progressive recovery in $CD4^+$ T-cell counts over many months, rather than a rapid rise over 4-6 weeks. A rapid rise after only 1 month of ART occurred for Patient 3; $CD4^+$ T-cell count rose from 249 cells/mm^3 1 month before treatment to 411 cells/mm^3 1 month after treatment started. Whilst the most significant rise in absolute $CD4^+$ T-cell count after 1 month of ART was seen for Patient 3, $CD4^+$ T-cell percentage gain by that point was greater for Patients 2, 4 and 5 than for Patient 3. This is due to the fact that absolute $CD8^+$ T-cell counts dropped significantly after 1 month ART for Patients 2, 4 and 5 (Patient 2: 760 to 536, Patient 4: 1429 to 1005, Patient 5: 892 to 421 cells/mm^3).

By the end of the observation period, a significant increase in absolute $CD4^+$ T-cell count and percentage was seen for Patients 3, 4 and 5. For Patients 1 and 2 the increase in absolute $CD4^+$ T-cell count was minimal, but Patient 2 experienced a recovery of $CD4^+$ T-cell percentage due to a decrease in total $CD8^+$ T-cell count. Longer follow-up of these patients may show further $CD4^+$ T-cell gains.

Table 2.3 $CD4^+$ T-cell values in five Discord Controllers following starting ART

	Change in $CD4^+$ T-cell count (cells/mm^3)	Change in $CD4^+$ T-cell %	Change in $CD4^+$ T-cell count (cells/mm^3)	Change in $CD4^+$ T-cell %	Total time observed on ART
	1 month ART		Total increase during time observed on ART		
Patient 1	+16	0	+45	+4	12 months
Patient 2	-23	+6	+50	+10	18 months
Patient 3	+162	+3	+165	+13	19 months
Patient 4	-6	+4	+244	+12	42 months
Patient 5	-1	+12	+157	+19	22 months

In summary, a cohort of 82 viraemic controllers was established comprising 64 typical controllers and 18 discord controllers with equivalent viral RNA-loads. The lower absolute $CD4^+$ T-cell count in discord controllers also represented a lower $CD4^+$ T-cell percentage suggesting specific depletion of these cells in this cohort. Epidemiological characteristics in each cohort were similar. No evidence was found for longer duration of HIV-1 infection in discord controllers as time since diagnosis and age were also indistinguishable comparing the groups. HIV-1 subtype distribution was similar comparing the controller cohorts, with a non-significant trend towards increased frequency of subtype C in discord controllers. Three of five discord controllers started on ART experienced slow but progressive $CD4^+$ T-cell recovery, with recovery of $CD4^+$ T-cell percentage but not absolute count in a further patient due to a decrease in total $CD8^+$ T-cell count.

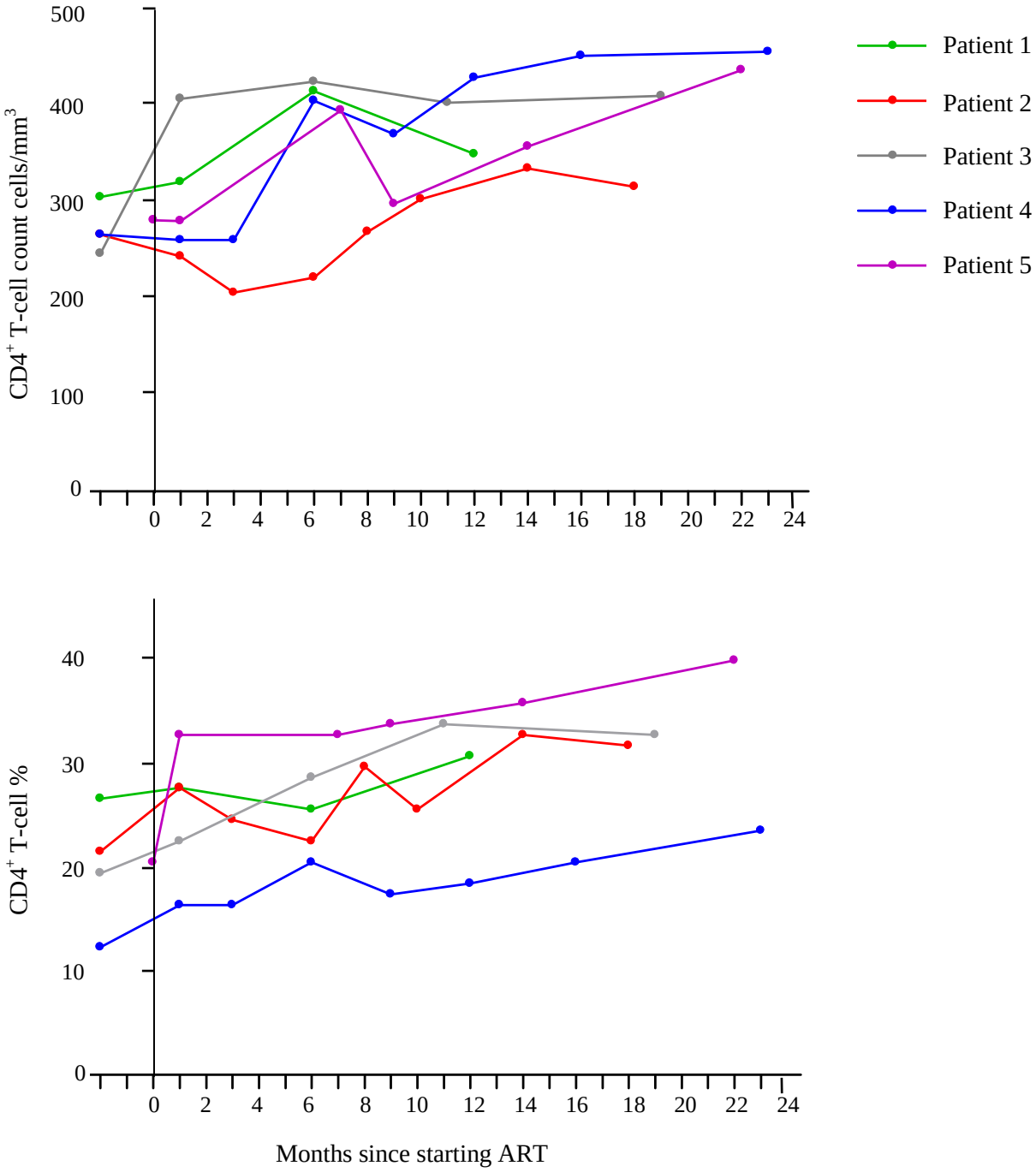


Figure 2.4 CD4$^+$ T-cell Count and Percentage in Five Discord Controllers Following Starting ART. CD4$^+$ T-cell values at the point of starting ART available for only patient 5. For the other patients the values shown before 0 months are those taken within three months prior to starting ART.

2.4 Discussion

The purpose of work described in this Chapter was to determine whether there were epidemiological or viral factors such as HIV-1 subtype which could distinguish discord controllers from typical controllers. Additionally, follow-up of discord controllers started on ART was undertaken to determine whether $CD4^+$ T-cell counts recovered on ART (which would further suggest initial low counts result from $CD4^+$ T-cell loss rather than a normal low level due to ethnic variation).

The cut-off of 450 $CD4^+$ T-cells/mm^3, used to distinguish typical from discord controllers, was selected based on the fact that many laboratories define a normal $CD4^+$ T-cell count to be above this value [326]. In addition, the 'When to start consortium' [157] have found decreased mortality is derived from starting ART in the range 351-450 cells/mm^3 rather than waiting for counts to drop below 350 cells/mm^3. This further supports the notion that $CD4^+$ T-cell counts below 450 cells/mm^3 are suboptimal.

All of the patients studied here have viral RNA-loads which are much lower than is usual without ART treatment for HIV-1. In the majority of these patients their immune system does not appear to be affected by this low level replication, as far as can be determined with routine laboratory surveillance: absolute $CD4^+$ T-cell counts and $CD4^+$ T-cell percentage. As a result these patients typically remain clinically well and do not require treatment with ART. This is in keeping with the fact that low viral RNA-loads usually predict more stable $CD4^+$ T-cell counts [243, 244]. However, it is clear from this study that a significant subset do suffer significant $CD4^+$ T-cell losses; indeed, 22% of the viraemic controller cohort were found to have suboptimal $CD4^+$ T-cell counts and therefore were categorised at the study outset as being discord controllers.

2.4.1 HIV-1 RNA-load and CD4$^+$ T-cell Values in Discord Controllers and Typical Controllers

Viral RNA-load set-point is associated with the rate of disease progression [243, 244, 327]. Whilst both discord controllers and typical controllers have controlled viral RNA-loads, it was important to determine whether viral RNA-loads were comparatively lower in typical controllers, which could be viewed as an explanation for higher CD4$^+$ T-cell counts. There were elite controllers in both groups, with a non-significant trend towards more in the typical controller cohort. This is in keeping with the fact that elite controllers have more stable overall CD4$^+$ T-cell count trends than do viraemic controllers [318]. Viral RNA-loads were found to be similar comparing the two groups (see Figure 4.2 b), suggesting that a relative difference here cannot to be proposed to account for lower CD4$^+$ T-cell counts in discord controllers.

To eliminate the effect of the possibility of varying efficiencies of different viral RNA-load assays on HIV-1 isolates of differing subtype, patients were not classed as viraemic controllers unless their viral RNA-load had been tested on at least two different assay platforms and found to have a viral RNA-load of <2000 copies/ml on each. PCRs designed to quantify HIV-1, including those used in this study, rely on nucleic acid-based amplification with detection of specific target sequences. Consequently, mismatches at primer and probe sites due to the genetic variability of HIV-1 have the potential to reduce the efficiency of hybridisation, resulting in the inability to detect viral RNA-load accurately [328]. Any single viral RNA-load assay platform will underquantitate a small percentage of the time given the inherent heterogeneity of HIV-1 species, hence the need to confirm an atypically low result using a second assay.

For three viraemic controllers, control of viral RNA-load was lost during follow-up. It is possible that this was a result of super-infection with another virus; this has previously been shown to be a cause of loss of control of viral RNA-load [329]. Sequential analysis of infecting virus during viraemic control and after control was lost would therefore have been of interest in these patients. It may also be the case that viral rebound was due to the development of viral escape mutations or loss of effective immune responses as has been described previously [330, 331]. This, together with the varying patterns of HIV-1 RNA-load control and CD4$^+$ T-cell homeostasis as shown in Appendix II, further highlights the fact that alternate, as yet undefined, factors play a significant role in control of viral RNA-load and CD4$^+$ T-cell homeostasis.

As discussed in Section 1.6.1, there remains some debate regarding whether absolute CD4$^+$ T-cell count or CD4$^+$ T-cell percentage is a more reliable marker of immune status in HIV-1

infected individuals. Since there is some ethnic variation in normal $CD4^+$ T-cell and total lymphocyte counts [332, 333] it could be argued that some patients may have a lower $CD4^+$ T-cell count on account of their ethnic group. In those patients one might expect to see low absolute $CD4^+$ T-cell counts but normal $CD4^+$ T-cell percentage given total lymphocyte count is also lower. In order to exclude this as a factor leading to spurious inclusion in the discord controller cohort, $CD4^+$ T-cell percentage was also assessed in each controller cohort. The fact that $CD4^+$ T-cell percentage was significantly lower in the discord controller group compared with typical controllers, together with the fact that ethnicity isn't significantly different comparing the two controller groups, suggests that the lower absolute $CD4^+$ T-cell counts in the discord controller group are not merely a feature of ethnic differences and a lower normal $CD4^+$ T-cell range, and that these low counts are a result of specific depletion of this cell type.

2.4.2 Epidemiological and Clinical Features of Discord Controllers and Typical Controllers

Epidemiological factors (age, sex, place of birth, ethnicity or risk behaviour) were found to be similar when comparing discord controllers and typical controllers. Therefore, this study did not identify a factor other than $CD4^+$ T-cell count which distinguished discord controllers from typical controllers. A higher proportion of the whole viraemic controller cohort were women than men as has been reported previously amongst viraemic controllers (definition used viral RNA-load <400 copies/ml) [319]. The reason for over-representation of women is not fully understood [319].

No association was found between the discord phenotype and time since first positive test (surrogate for time since seroconversion) suggesting that the discord controllers are not simply those viraemic controllers which have been infected for a longer time period when compared with typical controllers. This supposition is also supported by the fact there was no association between discord controller phenotype and age. Whilst time since diagnosis is not a perfect surrogate for time since seroconversion, this data does suggest that the discord controller phenotype is not associated with a longer duration of infection compared with typical controllers.

There was no evidence of increased opportunistic disease in discord controllers compared to typical controllers (Appendix III), despite their lower $CD4^+$ T-cell counts.

2.4.3 HIV-1 Subtypes in Discord Controllers and Typical Controllers

The HIV-1 subtype distribution found in the East London HIV-1 cohort studied here is far more diverse than is reported for the U.K. A study based on 5,675 *pol* gene sequences recorded in the United Kingdom Drug Resistance Database found 74.9% sequences to be subtype B, 10.0% were subtype C, 6.4% subtype A and 1.8% CRF02_AG amongst other less frequent subtypes [64]. In the East London HIV-1 population, as shown in Figure 2.3, only 35.7% are subtype B demonstrating that there is a far higher representation of non-B subtypes in the East London HIV-1 population compared with the U.K. in general. This is likely to be at least in part due to the high proportion of Black African people in the East London HIV-1 population; a study based in South London showed that non-B subtype isolates account for 96.8% of infections among Black Africans compared with only 14.2% in Caucasians [334].

There was a non-significant trend towards increased frequency of HIV-1 subtype C in the discord controllers compared with the typical controller cohort. Whilst studies have suggested increased pathogenicity of subtype D isolates [31], a similar rate of disease progression is observed in patients infected with subtype C infection when compared with subtypes A, B and CRF02_AG [31]. Therefore, relative pathogenicity of subtype C isolates does not appear to explain to any degree the increased frequency of this subtype in discord controllers.

A product for sequencing and subtype prediction was not obtained using either subtyping method for two viraemic controllers patients. Since subtype was not determined in all patients tested, it is possible that the subtype data may be skewed slightly by this.

2.4.4 Response of Discord Controllers to ART

In three out of five discord controllers who were treated with ART a clear gain in $CD4^+$ T-cell count was observed in keeping with those described by Okulicz *et al.*[322]. This further supports the notion that $CD4^+$ T-cell counts in the discord controller population were low as a result of depletion rather than a lower normal range in these patients. It is also of relevance to the clinician treating these patients to know whether $CD4^+$ T-cells recover in the same way that they do in non controllers. It is notable that, in keeping with the study by Okulicz et al., work here shows that $CD4^+$ T-cell gains were seen in these patients over a prolonged period, with gains continuing to be seen past 12 months of ART. Indeed, a significant early rise in $CD4^+$ T-cell count was seen only in one patient (Patient 3, Table 2.3, Figure 2.4). Where early increases in $CD4^+$ T-cell count after starting ART are seen, this has been proposed to be due to

redistribution of T-cells resulting from ART-mediated resolution of the immune activation that had sequestered $CD4^+$ T-cells within lymphoid tissues [167, 290], suggesting this may have been a significant factor for Patient 3.

Even though a significant early rise in absolute $CD4^+$ T-cell count was seen only in Patient 3, $CD4^+$ T-cell percentage increases were seen in three further patients after 1 month of ART because total $CD8^+$ T-cell counts had decreased significantly during that time. This has been demonstrated previously, with other studies reporting a decrease in either memory $CD8^+$ T-cells [335], activated $CD8^+$ T-cells [336] or HIV-1 specific $CD8^+$ T-cells [178] within the first few weeks of ART. It is thought that the reason for this is that suppression of viral RNA-load results in reduced antigen driven expansion of the $CD8^+$ T-cell compartment. Therefore, the early $CD4^+$ T-cell percentage increases in these three patients is likely to represent restoration of a more normal $CD8^+$ T-cell profile given the suppressed viral replication, rather than reconstitution of the $CD4^+$ T-cell pool. The fact that ART leads to restoration of a normal $CD8^+$ T-cell pool in discord controllers, suggests ART suppresses antigen driven expansion of the $CD8^+$ T-cell compartment in this cohort too. This suggests that there is high level HIV-1 replication in discord controllers which is not reflected in significant viral RNA-loads. This proposal is further supported by HIV-1 DNA load data which is presented in Chapter 4.

Four of the five patients started on ART were not elite controllers and thus had detectable viral RNA-loads. Viral RNA-load for all four patients went from being detectable to undetectable using current standard assays. It may be that this reduction in viral RNA-load, albeit from an already low level, is key to $CD4^+$ T-cell reconstitution. The fact that $CD4^+$ T-cell count gains were seen here, despite already low pre-treatment viral RNA-loads, supports current treatment guidelines which emphasise the need to initiate ART on the basis of $CD4^+$ T-cell count rather than viral RNA-load [337].

One study reported that an elite controller with progressive $CD4^+$ T-cell depletion did not experience $CD4^+$ T-cell reconstitution on ART [338]. This is in keeping with the fact that $CD4^+$ T-cell gains were minimal in the one elite controller (Patient 2) put on ART in this study. This same study reported a decrease in viral RNA-load from 7 to <1 copies/ml (using an ultrasensitive research assay) in the elite controller put on ART [338]. This might suggest there is a point at which viral RNA-load is so low, decreasing it further does not lead to $CD4^+$ T-cell reconstitution. It must also be noted that this study examined only one elite controller put on ART, and it also relies on accurate quantification of extremely low level viral RNA-load.

These results taken together suggest that although suppression of viral RNA-load is important in reconstitution of $CD4^+$ T-cell count in many patients (even where viral RNA-loads are already relatively low as in viraemic controllers with detectable viral RNA-load), there are additional factors involved in $CD4^+$ T-cell count reconstitution.

In summary, data presented here demonstrates that a significant subset of viraemic controllers have a low $CD4^+$ T-cell count, and suggests that it is low as a result of $CD4^+$ T-cell depletion rather than a low count which is normal for those individuals. These discord controllers appear epidemiologically similar to typical controllers and this study finds no evidence that discord controllers have been infected for a longer duration than typical controllers. This study found a trend towards a higher representation of the subtype C amongst discord controllers compared with typical controllers. Most discord controllers experience slow recovery of $CD4^+$ T-cell counts when put on ART, with early recovery of $CD4^+$ T-cell percentage due to a decrease in total $CD8^+$ T-cell counts.

CHAPTER 3

Characterisation of T-cell Populations in Discord Controllers and Typical Controllers

3.1 Introduction

In HIV-1 infection the mechanisms leading to depletion of peripheral $CD4^+$ T-cells are not fully defined. Mechanisms which have been proposed and are not mutually exclusive include: 1) apoptosis of uninfected $CD4^+$ T-cells as a result of chronic immune activation observed in progressive disease [263-265, 267, 339-341]; 2) impaired production of $CD4^+$ T-cells as suggested by the preferential depletion of naïve T-cells in progressive disease [265, 295, 296]; and 3) sequestration to another site within the body.

Work described in this Chapter was designed to determine the contribution of these first two factors to $CD4^+$ T-cell decline in discord controllers. Therefore, the hypothesis was that changes typically associated with disease progression, high level $CD4^+$ and $CD8^+$ T-cells activation and depleted naïve $CD4^+$ and $CD8^+$ T-cells, would be more marked in discord controllers as compared with typical controllers. Comparison of discord controllers with typical controllers provides the opportunity to distinguish immunological changes associated with disease progression from those which result from high viral RNA-load. This distinction is not possible with analysis of patients with the typical pattern of progression (high viral RNA-load, low $CD4^+$ T-cell count).

3.2 Methods

T-cell work was carried out on blood from seven discord controllers with a geometric mean $CD4^+$ T-cell count of ≤400 cells/mm^3, and from 12 typical controllers with a mean $CD4^+$ T-cell count of ≥500 cells/mm^3, in order to avoid those patients too near the 450 cells/mm^3 cut-off which distinguishes these two cohorts. For comparison, blood was also taken from five progressors (viral RNA-load > 10000 copies/ml, $CD4^+$ T-cell ≤400 cells/mm^3 (see Figure 2.1) and five uninfected patients.

3.2.1 T-cell Immunophenotyping using Flow Cytometry

Seven colour flow cytometry was used to quantify $CD4^+$ T-cell and $CD8^+$ T-cell populations and their naïve ($CD45RA^+CD62L^+$), central memory ($CD45RO^+CD62L^+$) and effector memory ($CD45RO^+CD62L^-$) subsets in freshly EDTA-anticoagulated blood. Activation level of a particular T-cell subset was defined as percentage activation where an activated cell expressed both CD38 and HLA-DR [342]. The following monoclonal antibodies and fluorochromes were used: CD3-Pacific Blue, CD4- allophycocyanin(APC)-H7, CD45RA-phycoerythrin (PE)-Cy7, CD45RO-phycoerythrin (PE)-Cy7, CD62L-PE, HLA-DR-peridinin chlorophyll protein (PerCP), CD38-PerCP-Cy5.5, CLA-FITC, β7 –APC (BD Biosciences, San Jose, CA).

The staining matrix allowing colour compensation on subsequent analysis is shown in Appendix I. Saturating amounts of monoclonal antibodies or their respective isotype controls accord to this matrix were incubated with 100µl of whole blood (within 4 hours of collection) for 15 minutes at room temperature. 500µl Optilyse C (Immunotech, Marseilles, France) was added and incubated at room temperature for a further 15 minutes. Blood aliquots with single colour staining were processed with each run for compensation. After two washes with phosphate-buffered saline containing 2% FCS, 0.02% NaN_3 and 1mM EDTA, stained cells were re-suspended and fixed in 300µl phosphate-buffered saline 4% paraformaldehyde. A constant volume of Flow-count Fluorospheres (Beckman Coulter, USA) was added to enable absolute quantification of cells. The cells were acquired within 12 hours of fixing on a BD LSR II cell analyser with a minimum of 50000 (usually 100000) events acquired per sample.

Efforts taken to preserve expression of surface molecules in T-cells include the use of only fresh blood samples which were processed within 24 hours of collection and the use of NaN_3 in the wash buffer which inhibits the potential redistribution of cell surface markers by either endocytosis or shedding.

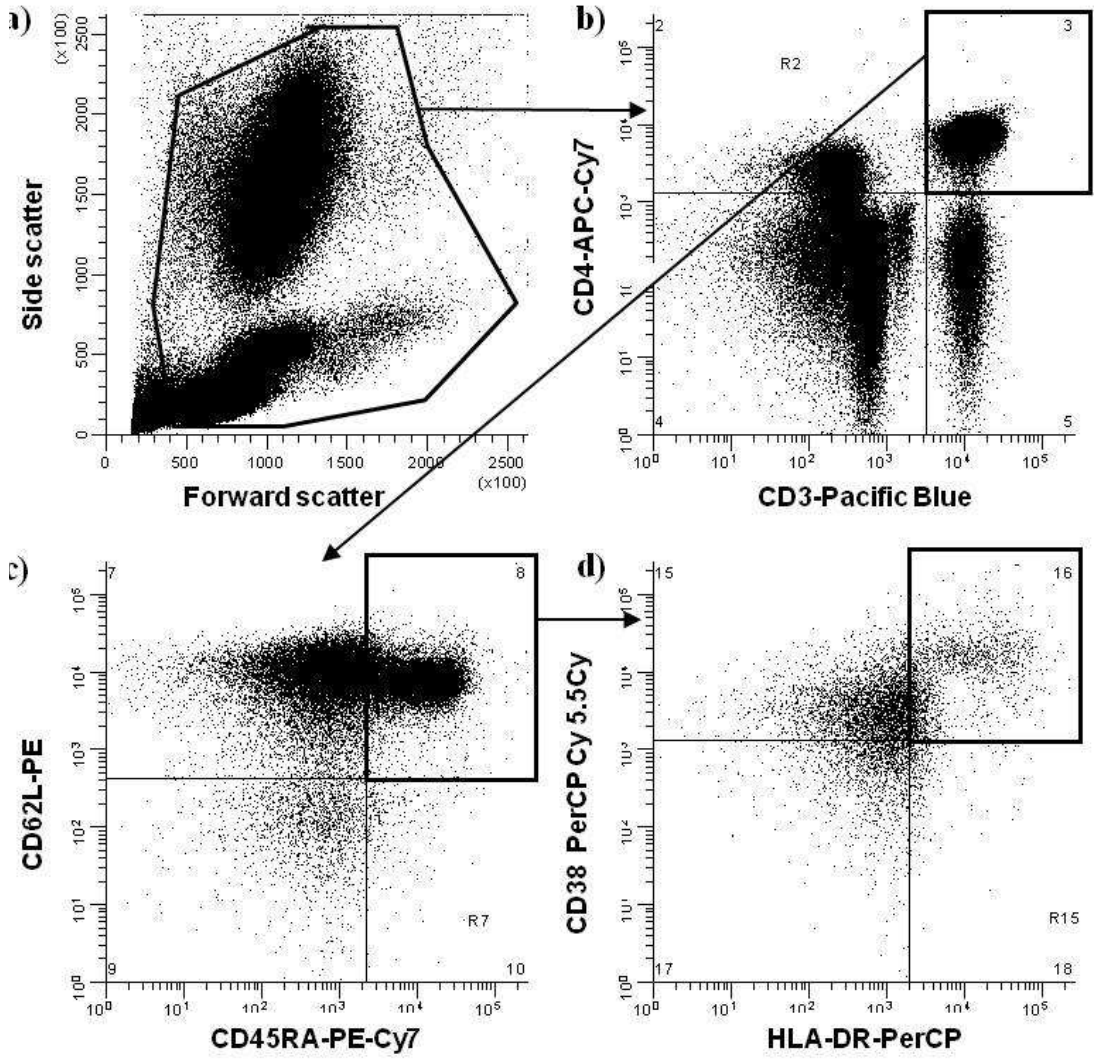


Figure 3.1 Gating strategy for flow cytometric analysis of T-cell subsets. Example given here for activated naïve $CD4^+$ T-cells. a) Forward and side scatter to define viable cell gate; b) Double staining of viable cells with anti-CD3 and anti-CD4. $CD4^+$ T-cells are in the top right quadrant; $CD4^-$ T-cells (predominately $CD8^+$ T-cells) are in the bottom right quadrant; c) Double staining of $CD4^+$ T-cells with anti-CD45RA and anti-CD62L, naïve $CD62L^+CD45RA^+$ T-cells, defined with reference to staining with isotype-matched control antibodies, are in the top right quadrant; d) Double staining of naïve $CD4^+$ T-cells with anti-CD38 and anti-HLA-DR, activated $CD38^+$HLA-DR^+ T-cells, defined with reference to staining with isotype-matched control antibodies, are in the upper right quadrant. The gating procedure was carried out to identify central memory $CD4^+$ T-cells ($CD3^+CD4^+CD45RO^+CD62L^+$) and effector memory $CD4^+$ T-cells ($CD3^+CD4^+CD45RO^+CD62L^-$), naïve $CD8^+$ T-cells ($CD3^+CD4^-CD45RA^+CD62L^+$), central memory $CD8^+$ T-cells ($CD3^+CD4^-CD45RO^+CD62L^+$), effector memory $CD8^+$ T-cells ($CD3^+CD4^-$ $CD45RO^+CD62L^-$). Within each population activated cells were defined as $CD38^+$HLA-DR^+.

Winlist 6.0 software (Verity Software House, Maine) was used for analyses and colour compensation. The gating strategy is shown in Figure 3.1. $CD4^+$ T-cells were identified within a viable cell gate, set on the basis of light scatter, as cells that were $CD3^+CD4^+$. $CD8^+$ T cells were identified as mononuclear cells that were $CD3^+CD4^-$. Two-dimensional dot plots, in which quadrant gates were set on the isotype controls, were used to define naïve, central memory and effector memory populations in both $CD4^+$ and $CD8^+$ populations. In turn, each of these populations were viewed on further 2-dimensional plots to determine the percentage of cell with an activated phenotype (co-expression of CD38 and HLA-DR). The number of flow-count spheres acquired allowed a precise determination of sample volume acquired allowing expression of number of cells per volume of blood.

3.2.2 Statistical Analysis

Statistical analysis and graphical presentations were performed using Prism (version 4.0, Graphpad). Differences in T-cell numbers and percentages in each cohort were assessed using a 2-tailed Mann-Whitney U test and results were considered significant if $p<0.05$. A correction for multiple comparisons was employed using the false discovery rate calculation. Given the small sample numbers, non-significant results were treated with caution.

3.3 Results

3.3.1 Patient Demographics and Laboratory Parameters

Demographics together with $CD4^+$ T-cell count and HIV-1 RNA-load distributions in HIV-1 infected patient groups included in T-cell characteristics experiments are shown in Table 3.1.

Table 3.1 Demographics, HIV-1 RNA-loads and **$CD4^+$ T-cell counts in patients tested for T-cell characteristics.**

	Discord Controllers (*n* = 7)	Typical Controllers (*n* = 12)	Progressors (*n* = 5)
Age, years	37 (21 - 63)	43.5 (30 - 53)	35 (30 - 48)
Female	86%	42%	40%
$CD4^+$ cells/mm^3	337 (267 - 400)	815 (691 - 971)	309 (296 - 339)
HIV-1 RNA-load copies/ml	675 (128 - 1320)	268 (116 - 926)	60,682 (28 603 – 15 5518)

Age, $CD4^+$ T-cell counts and HIV-1 RNA-loads are expressed as median (range).

3.3.2 $CD4^+$ T-cell Subsets

As expected, the total cell numbers were reduced for all $CD4^+$ T-cell populations in progressors and discord controllers compared with typical controllers (Figures 3.2 I, a,b,c). Similar to progressors, discord controllers had preferential depletion of naïve $CD4^+$ T-cells compared with typical controllers as shown by a reduction in the percentage of total T-cells with a naïve phenotype (Figures 3.2 I, d,e,f). The higher percentage of effector memory cells (Figure 3.2 I, f) in discord controllers and progressors is a reciprocal change due to preferential loss of naïve cells (as shown by lower absolute numbers; Figure 3.2 I, c). The percentage of central memory cells in blood was similar in all three patient groups (Figure 3.2 I, e).

3.3.3 CD4$^+$ T-cell Activation

Progressors showed increased levels of activation in naïve, central memory and effector memory CD4$^+$ T-cell subsets (Figures 3.2 I, g,h,i). In the discord controllers, all subsets of CD4$^+$ T-cells tended towards higher levels of activation similar to that seen in progressors. In contrast, typical controllers had low activation levels, indistinguishable from that seen in uninfected subjects, apart from a small but significant increase in the frequency of activated CD4$^+$ effector T-cells.

In summary of the CD4$^+$ T-cell compartment, discord controllers show changes not seen in typical controllers but which are similar to those seen in progressors: preferential depletion of naïve CD4$^+$ T-cells and increased CD4$^+$ T-cell activation in all subsets.

3.3.4 CD8$^+$ T-cell Subsets

Unlike progressors but similar to uninfected individuals, typical and discord controllers both maintain high levels of naïve CD8$^+$ T-cells (Figures 3.2 II, a,d). Progressors have a preferentially depleted naïve CD8$^+$ T-cell compartment (reduced numbers and percentage of total) and have expanded numbers of effector memory CD8$^+$ T-cells compared with all other patient groups (Figure 3.2 II, f).

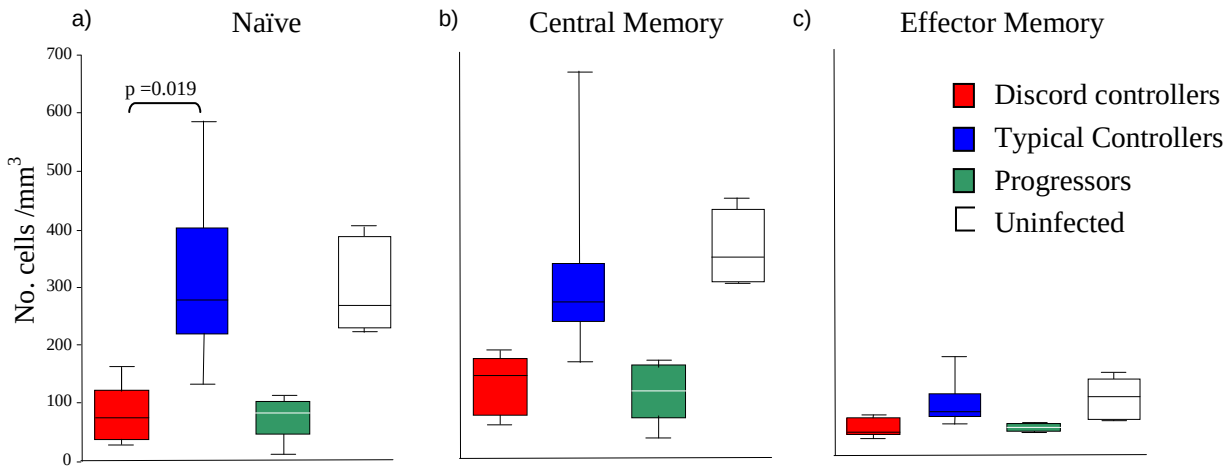
3.3.5 CD8$^+$ T-cell Activation

Higher activation levels were seen in all CD8$^+$ T-cell subsets in all HIV-1 infected groups compared with uninfected controls (Figures 3.2 II, g,h,i). CD8$^+$ T-cell activation levels in discord controllers were most similar to those seen in typical controllers in all CD8$^+$ T-cells populations (Figures 3.2 II, g,h,i). Thus, despite the fact that discord controllers share low total CD4$^+$ T-cell counts with the progressors, their CD8$^+$ T-cell activation pattern more closely resembles that of typical controllers.

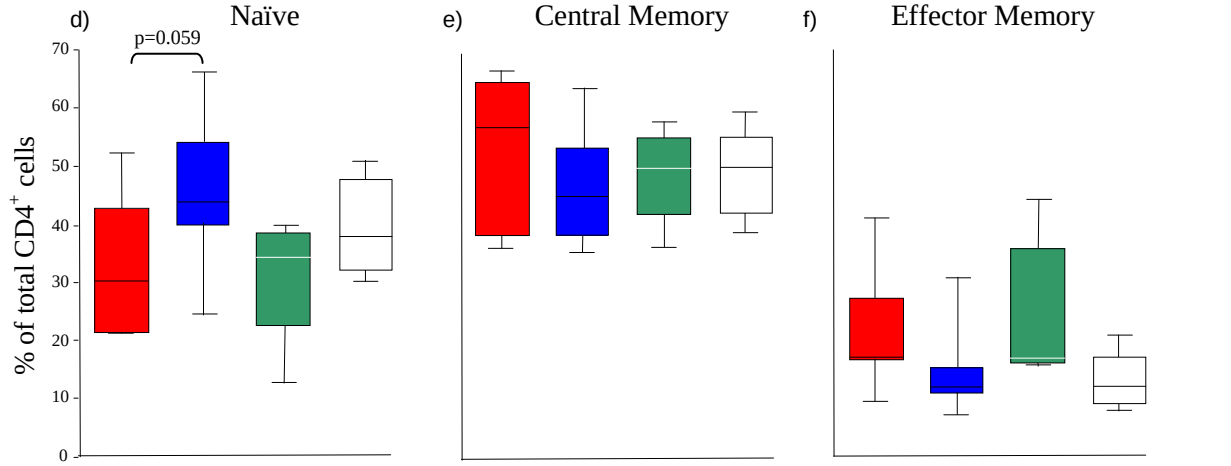
In summary of the CD8$^+$ T-cell compartment, discord controllers appear similar to typical controllers and dissimilar to progressors, demonstrating preserved naïve CD8$^+$ and only low level CD8$^+$ T-cell activation.

Absolute number of CD4$^+$ T-cells in each Subset

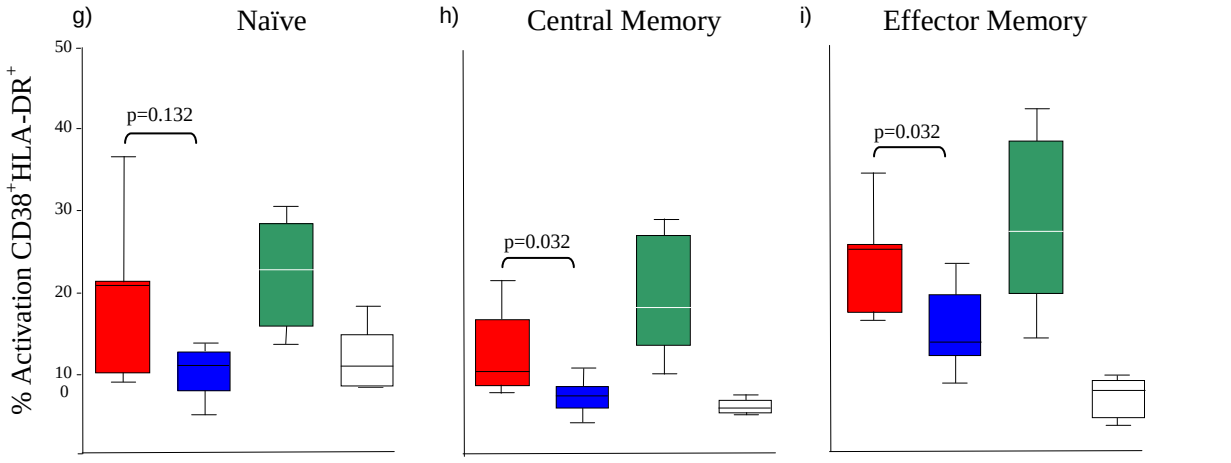
I



Percentage of Total CD4$^+$ T-cells

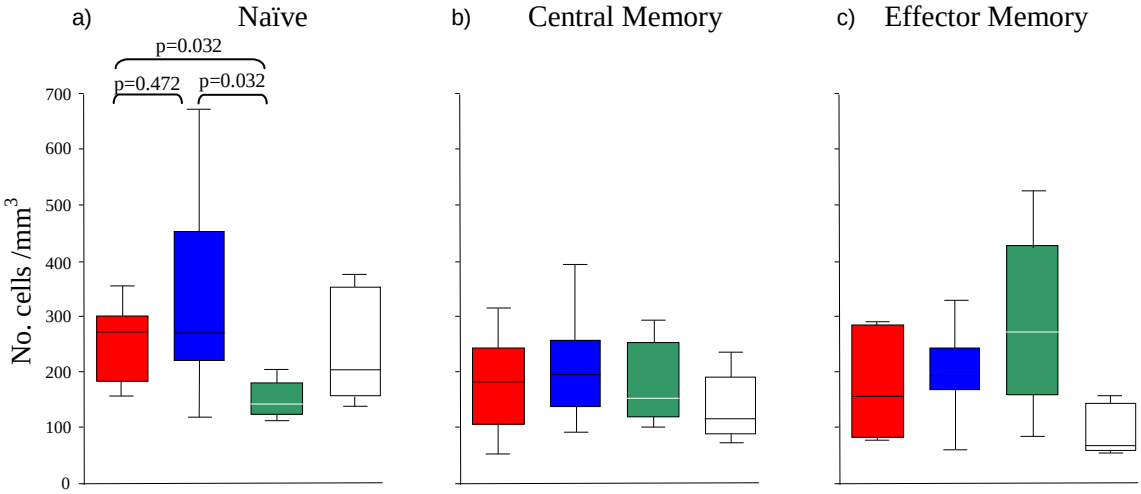


Percentage Activation

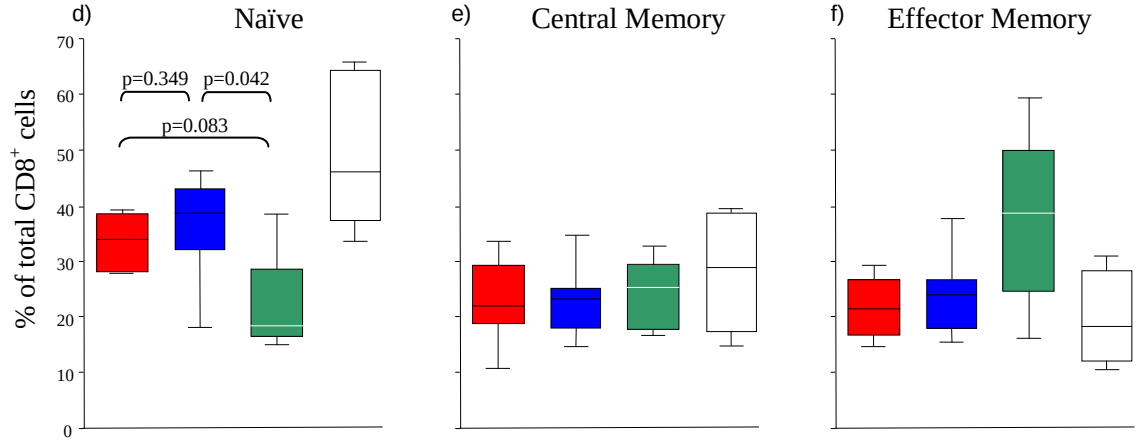


II

Absolute number of CD8$^+$ T-cells in each Subset



Percentage of Total CD8$^+$ T-cells



Percentage Activation

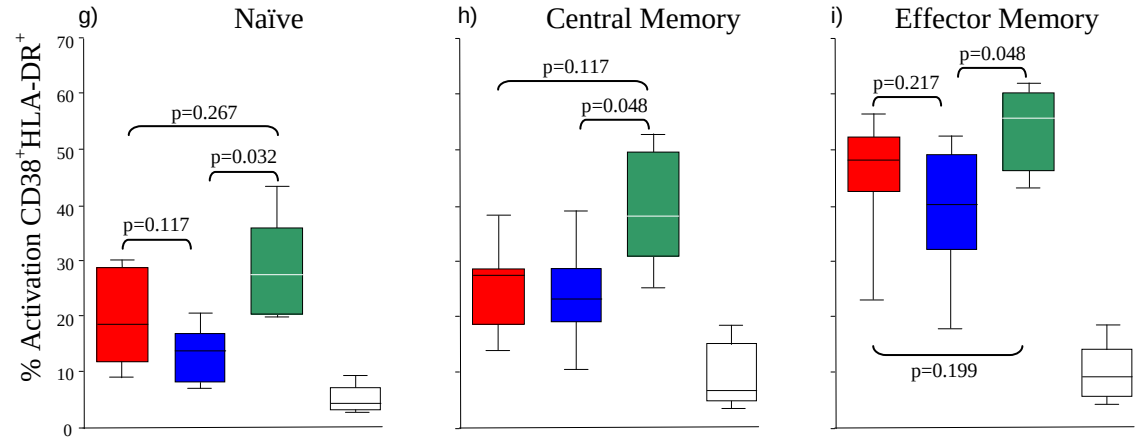


Figure 3.2 Representation of $CD4^+$ T-cell subsets and percentage activation in Discord Controllers and Typical Controllers in, I, $CD4^+$ T-cells and, II, $CD8^+$ T-cells. Patients analysed included discord controllers and typical controllers, together with progressors and uninfected subjects for comparison. Absolute numbers of cells in each of $CD4^+$ and $CD8^+$ T-cell subsets are shown in a) Naïve ($CD45RA^+CD62L^+$), b) central memory ($CD45RO^+CD62L^+$), and c) effector memory ($CD45RO^+CD62L^-$). Percentage of cells in each of $CD4^+$ and $CD8^+$ T-cell subsets compared with total $CD4^+$ or $CD8^+$ T-cell pool are shown for d) Naïve, e) central memory, and f) effector memory**.** Percentage activation of cells in $CD4^+$ T-cell subsets (as percentage of total cells in that subset) by patient group is shown in g) - i). Each central bar represents the median value, each box represents the IQR and the whiskers represent the minimum and maximum values. Relevant p values are displayed.

3.3.6 T-cell Characteristics in a Patient Subsequently Started on ART

Although not a specific aim of this work, it was of interest to observe T-cell parameters in one discord controller patient (Patient 3) who subsequently started on ART to see if there were any suggestions of parameters that might distinguish this individual from those who did not need to start ART.

In this patient naïve $CD4^+$ T-cell count was 120.3 cells/mm^3, which was 40.4% of total $CD4^+$ T-cells. The percentage of activated naïve $CD4^+$ T-cells was 20.5%, in central memory 12.2% and in effector memory 21.6%. Naïve $CD8^+$ T-cell count was 273.4 cells/mm^3, which was 36.8% of total $CD8^+$ T-cells. The percentage activation was 18.8% in naïve $CD8^+$ T-cells, 26.3% in central memory $CD8^+$ T-cells and 48.0% in effector memory.

Comparing these results to those seen in Figure 3.2, values look typical for the discord controller group, except that central and effector memory $CD4^+$ T-cell activation levels appear to be at the lower end of the spectrum.

3.4 Discussion

Both chronic immune activation of $CD4^+$ and $CD8^+$ T-cells [263-265, 267, 339-341] and preferential depletion of $CD4^+$ and $CD8^+$ naïve T-cells have been reported to be associated with progressive disease [265, 295, 296]. Viraemic controllers typically experience significantly longer times to progression than non controllers [318]. In keeping with this, immune activation has been reported to be lower in elite controllers than in non controllers [320, 343]. However, as described here, there is a population of viraemic controllers who do experience disease progression with depletion of $CD4^+$ T-cell counts, discord controllers, and as such viral replication and progression appear dissociated. It was therefore of interest to determine whether discord controllers displayed those features typically associated with disease progression. The hypothesis for this part of the work was that immunological features typically associated with disease progression (selective depletion of naïve $CD4^+$ T-cells, $CD4^+$ and $CD8^+$ T-cell activation) would be more marked in discord controllers than in typical controllers, and may therefore account for their $CD4^+$ T-cell depletion.

The results described here demonstrate that for the $CD4^+$ T-cell compartment the hypothesis may be accepted since discord controllers demonstrated a selective depletion of naïve $CD4^+$ T-cells and increased numbers of activated $CD4^+$ T-cells compared with typical controllers. These $CD4^+$ T-cell features in discord controllers were similar to those found in the progressors. It therefore appears that these immunological features are associated with $CD4^+$ T-cell decline in viraemic controllers and may account for the depleted $CD4^+$ T-cell counts seen in discord controllers.

In contrast, the discord controllers were similar to typical controllers when observing the $CD8^+$ T-cell compartment with a preserved naïve $CD8^+$ T-cell pool and relatively low levels of $CD8^+$ T-cell activation. Progressors displayed a depleted naïve $CD8^+$ T-cell pool and higher levels of $CD8^+$ T-cells activation compared with both controller groups. So here the hypothesis must be rejected since depletion of the naïve $CD8^+$ T-cell subset and/or high levels of $CD8^+$ T-cells activation were not seen in discord controllers, suggesting that these immunological features are not associated with $CD4^+$ T-cell decline in viraemic controllers.

The fact that the hypothesis regarding the $CD8^+$ compartment was refuted is at odds with other studies which suggest that $CD8^+$ T-cell activation is a predictor of $CD4^+$ T-cell decline, independent of $CD4^+$ T-cell count and HIV-1 RNA-load [267, 340, 344]. Instead, results presented here support alternate studies in which higher viral RNA-load positively correlates with increased $CD8^+$ but not with $CD4^+$ T-cell activation [264, 345], and that this correlation

holds regardless of $CD4^+$ T-cell counts [345]. This would predict, as was found in this study, low $CD8^+$ T-cell activation levels in discord controllers despite their low $CD4^+$ T- cell counts. Results described here show HIV-1 infected groups, including typical controllers, to demonstrate significantly higher $CD8^+$ T-cell activation in all compartments than in uninfected subjects. By contrast, $CD4^+$ T-cell activation was found to be similar in typical controllers compared with uninfected subjects. Hunt *et al.* report results which differ in respect to the $CD4^+$ T-cell compartment in that 30 elite controllers (defined as viral RNA load <75 copies/ml) were found to have higher $CD4^+$ and $CD8^+$ T-cell activation than uninfected subjects [320]. The reason for the difference in this result may be because the elite controller group described by Hunt *et al.* were not defined by their $CD4^+$ T-cell counts, thereby having a range of counts (350-642 cell/mm^3); results in their elite controller group may therefore have been skewed by high $CD4^+$ T-cell activation in those elite controllers with low $CD4^+$ T-cell counts. Indeed, they do additionally report a negative correlation between T-cell activation and $CD4^+$ T-cell counts in elite controllers; whilst this correlation is seen for both $CD4^+$ and $CD8^+$ T-cell populations, the correlation appeared stronger (Spearman's $\rho = -0.52$) and more significant for $CD4^+$ activation ($p = 0.009$) than for $CD8^+$ activation (Spearman's $\rho = -0.37$, $p = 0.047$), where the correlation was weak and only just reached significance. These findings do appear in keeping with results reported in this thesis, since in viraemic controllers $CD4^+$ T-cell decline appears more closely associated with $CD4^+$ T-cell activation than with $CD8^+$ T-cell activation.

Since work described in this thesis began, a few other investigators have examined T-cell populations in small numbers of viraemic controllers. Andrade *et al.* describe a single HIV-1 elite controller (<50 copies/ml) who experienced a progressive $CD4^+$ T-cell decline despite maintaining undetectable viral RNA-load. This patient was HLA B5801 positive and thought to be infected with a subtype B virus. The level of $CD4^+$ and $CD8^+$ T cell activation in this patient was found to be much higher than that seen in 10 elite controllers who maintained normal $CD4^+$ T-cell counts [321]. Sedaghat *et al*. observed $CD4^+$ T-cells counts in 8 elite controllers, and found that 3 experienced $CD4^+$ T-cell depletion. They found no relationship between the level of activation of $CD4^+$ or $CD8^+$ T-cells and $CD4^+$ T-cell depletion, but stated this might be due to small patient numbers [338]. Kamya *et al.* studied the slope of $CD4^+$ T-cell counts over time in 25 elite controllers (<50 copies/ml). The slope was not significantly different from 0 in 15 of these patients, but was positive in 3 and negative in 7 of them. Those with increasing and decreasing slopes were found not to differ based on $CD8^+$ T-cell activation levels, with the authors concluding that $CD8^+$ T-cell activation was not the cause of $CD4^+$ T-cell decline [346]. So in summary Hunt *et al.* found both $CD4^+$ and $CD8^+$ T-cell activation to be associated with $CD4^+$ T-cell decline in elite controllers (although the association was weak for $CD8^+$ T-cell

activation), Andrade *et al.* found both $CD4^+$ and $CD8^+$ T-cell activation to be associated, Sedaghat *et al.* found neither to be associated and Kamya *et al.* found $CD8^+$ T-cell activation not to be associated with $CD4^+$ T-cell decline in elite controllers. The published results are hence not in agreement highlighting the need for further studies on larger cohorts.

A study earlier this year reported naïve $CD4^+$ T-cell assessment in a group of 20 elite controllers with high $CD4^+$ T-cells counts and 5 with low $CD4^+$ T-cell counts, and compared them to non controllers with progressive disease. They demonstrated reduced proportions of naïve $CD4^+$ T-cells in elite controllers, even in those maintaining normal total $CD4^+$ T-cells counts; this depletion resembled that seen in non controllers. Those elite controllers with normal total $CD4^+$ T-cells counts were found to differ from those with low counts due to preserved thymic function [347]. This conflicts with data presented here where viraemic controllers with normal $CD4^+$ T-cell counts had a preserved naïve $CD4^+$ T-cell compartment, again highlighting the need for further studies.

It was not possible to thoroughly examine $CD4^+$ T-cell response to treatment as predicted by baseline $CD4^+$ T-cell activation as it was not often possible to obtain blood samples leading up to ART initiation. Consequently T-cell data was available for only one patient (Patient 3 from Table 2.3 and Figure 2.4) who subsequently started ART. In this patient a significant early increase in $CD4^+$ T-cell count was seen on ART. This early increase has been proposed to be due to redistribution of $CD4^+$ T-cell into the blood from the lymphoid tissues resulting from ART-mediated resolution of the immune activation [167, 290]. Contrary to findings here this might predict high $CD4^+$ T-cell activation in this patient. However, a study by Mildvan *et al.*, showed that baseline $CD4^+$ T-cell activation itself did not predict $CD4^+$ T-cell recovery in response to treatment [348], potentially explaining why $CD4^+$ T-cell activation was not higher in this patient. It should be noted that ART-mediated resolution of immune activation might act at the lymphocyte effector site (e.g. GI tract lamina propria) which might explain why levels of activation in circulating lymphocytes might not predict response. Similar studies on a greater number of patients would be needed to make any meaningful interpretation.

A potential limiting factor is the small numbers of discord controllers studied. This was in part due to the infrequent nature of the patients themselves, and also due to the complexity of the experiments. Another limitation is that that only a single snapshot for each patient was obtained; changes in these T-cell parameters over time in relation to HIV-1 RNA-loads and $CD4^+$ T-cells counts would be of interest. In addition, subset delineation using one or two surface markers is not perfect: for example not all CD45RA positive T-cells are naïve (since there is some reversion of CD45RO to CD45RA) [349].

In summary, this study demonstrates that compared with typical controllers, discord controllers demonstrate preferential depletion of naïve $CD4^+$ T-cells and increased activation of all $CD4^+$ T-cell subsets probably accounting for $CD4^+$ T-cell loss. $CD8^+$ T-cell parameters in discord controllers are indistinguishable from those in typical controllers. In contrast, progressors have marked naïve $CD8^+$ T-cell depletion and $CD8^+$ T-cell activation. It appears that maintenance of the $CD8^+$ T-cell compartment is associated with low viral RNA-loads. In conclusion, the $CD4^+$ T-cell compartment but not the $CD8^+$ T-cell compartment of discord controllers is typical of patients with progressive disease.

CHAPTER 4

Quantitative HIV-1 DNA-Loads in Discord Controllers and Typical Controllers

4.1 Introduction

The HIV-1 burden in the blood of infected patients can be measured as viral RNA contained in virions free in the plasma (viral RNA-load) or cellular viral DNA (viral DNA-load). In HIV-1 infection, cellular viral DNA-load is composed of different forms reflecting different stages during viral replication in the cell: the linear non-integrated form, the circular non-integrated form and the integrated provirus; the latter is the form from which transcription occurs [88].

Quantification of the viral RNA-load form in plasma by PCR has long been the chosen method used to monitor viral replication and predict progression both in the research setting and in the clinic [244, 315]. In viraemic controllers viral RNA-load is persistently low and most patients maintain normal $CD4^+$ T-cell counts [304]. However, as is described in this thesis, some viraemic controllers have low $CD4^+$ T-cell counts (discord controllers) and so demonstrate disease progression despite their low viral RNA-load; these low viral RNA-loads are similar to those found in typical controllers in whom $CD4^+$ T-cell counts are normal (see Table 2.1). The results in Chapter 3 suggest that the $CD4^+$ T-cell depletion is associated with activation of $CD4^+$ T-cells, even when there is lack of obvious viral replication in the blood as reflected in the viral RNA-load, as seen in discord controllers. It was not clear, however, based on viral RNA-load measurement alone, whether there are in fact higher levels of HIV-1 replication in a concealed or cryptic site which does not give rise to high viral RNA-load. This concealed or compartmentalised replication may play a direct role in $CD4^+$ T-cell depletion or may drive immune activation which leads to $CD4^+$ T-cell depletion. Thus the hypothesis for this Chapter is that, despite the lack of evidence of viral replication as suggested by low viral RNA-load, viral replication continues to be associated with $CD4^+$ T-cell depletion in viraemic controllers.

In order to address this hypothesis it was necessary to examine another marker of viral replication. HIV-1 DNA-load has been proposed as an alternate marker for ongoing replication *in vivo*, based on findings such as the fact that it is a marker for disease progression independent of viral RNA-load [350, 351]. Other studies demonstrate that viral DNA-loads are maintained by viral replication because they are suppressed by ART [352, 353] and that a significant correlation was observed between HIV-1 DNA load and unspliced RNA transcripts in patients on ART with undetectable viral RNA-loads [354]. Using HIV-1 DNA load as an alternate marker for viral replication, the stated hypothesis would predict that discord controllers have higher viral DNA-loads than typical controllers. Quantitative HIV-1 DNA-load assays have been reported previously, but before analysis of patient samples, a number of these were selected to evaluate further and determine the most appropriate method for the study.

The first part of this work is therefore the development and validation of a quantification HIV-1 DNA-load assay for use on PBMC samples from viraemic controllers (Sections 4.2 and 4.3). Subsequent Sections describe the use of the assay on blood from discord controllers and typical controllers (together with HIV-1 non controllers for comparison) to determine whether viral DNA-load correlates with $CD4^+$ T-cell decline.

4.2 Development of a Quantitative HIV-1 DNA-Load Assay

Since sensitive quantification of HIV-1 DNA was crucial, it was decided to design a Taqman real-time quantitative PCR assay (qPCR) as with careful assay design it provides sensitive and reproducible results with a wide dynamic range.

Given the worldwide increasing spread of HIV-1 genetic variants, it is mandatory that nucleic acid assays detect all HIV-1 subtypes. This is particularly important for this study because of the high prevalence of non-B subtypes in the East London HIV-1 cohort. Two primer-probes sets targeting HIV-1 LTR and HIV-1 Integrase were selected for assessment using qPCR following work which had previously been carried out (Barts and the London NHS Trust Diagnostic Virology Laboratory). Primers and probes binding highly conserved regions of the HIV-1 genome were assessed using conventional PCR methods which demonstrated their efficacy on a wide variety of HIV-1 subtypes.

It was anticipated that viral DNA-loads could be very low in viraemic controllers [314]. Therefore, a method based on initial recovery and concentration of PBMCs was used, as used by other groups [313, 353, 355], to enhance sensitivity of the assay.

Since most HIV-1 in the blood is associated with $CD4^+$ T-cells, and because $CD4^+$ T-cell counts are inherently variable in this population group, it was decided to report results as viral DNA copies/10^6 $CD4^+$ T-cells rather than viral DNA copies/10^6 PBMCs (composed of both lymphocytes and monocytes). The assay was designed to quantify total HIV-1 DNA-load (as opposed to integrated proviral DNA or unintegrated viral DNA) since in HIV-1 infection there is good evidence to suggest total HIV-1 DNA-load is a good marker of on-going viral replication [313, 350, 352, 353, 356].

Therefore, the aim of this part of the work was to develop a qPCR for quantification of total viral DNA-load in PBMCs, which would then be reported as HIV-1 DNA copies/10^6 $CD4^+$ T-cells.

4.2.1 Construction of a Standard Curve for HIV-1 LTR and HIV-1 Integrase qPCR

The HIV-1 primer and probe sequences used for development of the viral DNA-load assay are shown in Table 4.1. Both probes were labelled with a FAM (6-carboxy-fluorescein) reporter dye.

Table 4.1. Primer and probe sequences for HIV-1 LTR and HIV-1 Integrase qPCR

HIV-1 LTR	Forward Primer	5' - GCCTCAATAAAGCTTGCCTTGA [357]
	Reverse Primer	5' - GGCGCCACTGCTAGAGATTTT [358]
	Probe	5' - AAGTAGTGTGTGCCCGTCTGT [359]
HIV-1 Integrase [360]	Forward Primer	5' - GGTTTATTACAGGGACAGCAGAGA
	Reverse Primer	5' - ACCTGCCATCTGTTTTCCATA
	Probe	5' - ACTACTGCCCCTTCACCTTTCCAGAG

A plasmid dilation series was needed in order to construct a standard curve for target copy quantification. Products from conventional PCR of HIV-1 LTR and HIV-1 Integrase were TA-cloned, screened with subsequent purification of high copy number plasmid containing the relevant insert. (see Appendix I). One of each set was selected, and their concentrations established by spectrophotometry (NanoDrop spectrophotometer, used according to manufacturer's instructions). Using the DNA concentration determined using the Nanospot and the molarity of the plasmid with insert an expected copy number was calculated. From this a 10-fold dilution series was created, (1 = neat, 2 = 1 in 10 dilution, etc.), and dilutions 5 -12 were tested using the assays under development.

The HIV-1 LTR assay was assessed initially. The final 25µl PCR mixture contained 2×PCR QuantiTect Multiplex RT-PCR No Rox Mix (Qiagen), 0.2µM of each primer and 0.2µM probe. Thermocycling on the ABI 7500 Realtime PCR machine were 95°C for 15 minutes for activation of the Taq DNA polymerase; then 40 cycles of 95°C for 15 seconds and 52°C for 1 minutes. 5µl of plasmid dilution were added to duplicate wells for dilutions 5 to 11. 17 reactions of the 12^{th} dilution were prepared. Amplification reaching the threshold was seen in dilutions 5 to 11; for dilution 11, which contained an expected 4 copies per reaction, C_t values of 36.07 & 40.01 were obtained. For the 12^{th} dilution with a predicted 0.4 copies per reaction, 4

of 17 signalled, which is in keeping with the predicted copy number. This PCR was further optimised by increasing the anneal-elongation temperature from 52°C, as suggested by Luo *et al.* [359], to 60°C. This improved PCR efficiency giving a standard curve slope of -3.39.

The HIV-1 Integrase assay was analysed in a similar fashion with 10-fold dilutions. PCR mixture and conditions were as described for the HIV-1 LTR assay. The final dilution to amplify was the 10^{-11} dilution, for which it was expected there would be 2.9 copies per reaction based on molarity calculations. Twenty replicates of the 12^{th} dilution were then tested, and seven were found to signal again in keeping with the predicted 0.29 copies per reaction.

Dilutions 7 to 11 were selected for routine use for standard curve construction. This was because the copy number these dilutions represented was in the region of expected HIV-1 viral DNA-loads based on reports in the literature and because these dilutions also gave C_ts which were in the middle of the range (15-30).

The five plasmid dilutions for construction of the standard curve for the HIV-1 LTR and HIV-1 Integrase assays were run in duplicate. The ABI prism 7500 SDS 1.2 programme was then used to construct a standard curve for each assay. A typical example of a standard curve generated for the HIV-1 LTR assay is shown in Figure 4.1.

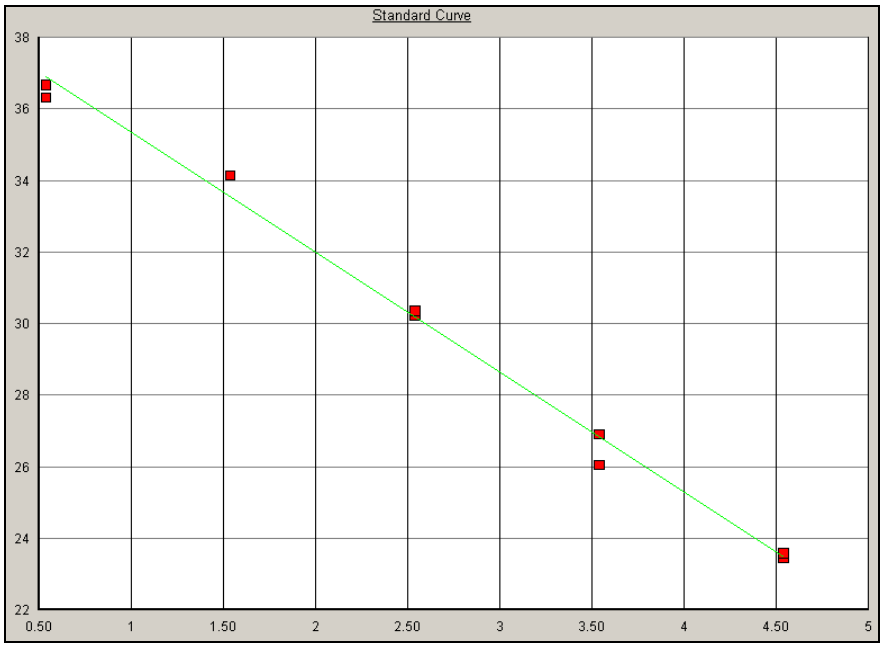


Figure 4.1 Example standard curve for HIV-1 LTR assay.

The efficiency of the assay was calculated using the formula:

efficiency = $(10^{-1/slope} -1) \times 100$

Hence the standard curve shown in Figure 4.1 for that particular HIV-1 LTR assay has an efficiency of 98.4%.

For all subsequent assays, as is recommended for qPCR, standard curve r^2 values were maintained at ≥0.99 ensuring good linearity and efficiency was maintained at 90-110% .

4.2.2 Sensitivity of the HIV-1 LTR and HIV-1 Integrase qPCRs on Patient Samples

Five blood samples from HIV-1 infected individuals were selected for testing with the HIV-1 LTR and HIV-1 Integrase qPCRs. So that viral DNA copy number could be expressed as per cell equivalent, a qPCR for the cellular house-keeping gene β-globin was run in parallel (this gene was selected since it is known there are always two copies per cell). This qPCR, which was adapted [361] from a previously published assay [362], used 0.4μM of each primer (Forward, 5' – GTGCACCTGACTCCTGAGGAGA, Reverse, 5' - CCTTGATACCAACCTGCCCAG) and 0.1μM FAM reporter dye (6-carboxy-fluorescein) labelled probe (5' –AAGGTGAACGTGGATGAAGTTGGTGG). A standard curve was developed as part of the work described here by creating a dilution series of high copy number plasmid with the PCR product insert in the same way as for HIV-1 LTR and HIV-1 Integrase assays (again r^2 values were ≥0.99 and efficiency 90-110%).

Since the sensitivity of the assay was important, PBMCs were first isolated from the blood samples. EDTA blood samples from five HIV-1 infected patients were centrifuged at 200g for 10 minutes at room temperature. Pastettes were used to aspirate the buffy coat from each and DNA was extracted using the Qiagen EZ1 biorobot and DNA Tissue programme according to the manufacturer's instructions into a 200μl eluate (6 water samples were also included as negative extraction controls). An internal inhibition control is important when employing PCR for use on blood due to the frequent presence of PCR inhibitors. For this purpose a fixed concentration of phocine herpes virus (PhHV) was added to each sample before extraction so that its subsequent detection using a parallel qPCR would provide an internal amplification control.

5μl of the extracted DNA was analysed in duplicate for the four separate qPCRs: HIV-1 LTR, HIV-1 Integrase, β-globin and PhHV. In addition, six test wells were used for negative extraction controls (NEC) and two wells for water as no template controls (NTC); these controls

were used in all subsequent experiments too. The PhHV qPCR run in parallel used the following primers: Forward, 5' – CGTTCCAACAACACAACCTACTG, Reverse, 5' - CTCTCATATCATCATTCAACTCAGTGT; and probe : 5'-ACCACCAATTACTCCTAGTCCACCACCG. The probe was labelled with a JOE reporter dye (6-carboxy-4', 5'-dichloro-2', 7'-dimethoxy-fluorescein).

Mean C_t values obtained from test wells were then compared with the standard curve to determine copy number. The results of the qPCR for the five patient samples are presented in Table 4.2 and show good correlation between HIV-1 copy number as assessed by the HIV-1 LTR and HIV-1 Integrase qPCRs. The PhHV C_t values were found to be within range for all samples (acceptable range 33.5 – 35.5 C_t) suggesting no significant qPCR inhibition.

Table 4.2. Target copy number in blood from five patients using HIV-1 LTR and HIV-1 Integrase and β-globin qPCRs

Sample	HIV-1 LTR	HIV-1 Integrase	β-globin
1	4.4	8.12	$3.19x10^8$
2	58.67	46.41	$3.85x10^8$
3	114.31	122.2	$6.76x10^8$
4	34.16	11.18	$6.66x10^8$
5	62.79	26.5	$4.07x10^8$
NTC & NEC	ND	ND	ND

NTC – no template control. NEC – negative extraction control. ND – not detected.

A further experiment designed to assess relative sensitivity of the two HIV-1 qPCRs used a sample with a very high viral RNA-load (1782250 copies/ml). The buffy coat layer was removed and from this a 5-fold dilution series in PBS was prepared. The neat buffy coat with four dilutions and a negative control underwent DNA extraction using the Qiagen EZ1 biorobot as described in Section 4.2.2. The result of this experiment, as shown in Table 4.3, showed that the HIV-1 LTR qPCR appeared to have superior sensitivity. For this reason, the LTR set was selected for use for all subsequent experiments, together with the qPCR for β-globin and PhHV.

Table 4.3. Target copy number in dilution series of PBMCs from blood of patient with high HIV-1 RNA-load using HIV-1 LTR, HIV-1 Integrase and β-globin qPCRs

Sample	HIV-1 LTR	HIV-1 Integrase	β-globin
Neat Blood	9.4	2.8	$2.3x10^6$
5^{-1}	0.5 & 0	0.88	$5.8x10^5$
5^{-2}	0.5 & 0	ND	$8.6x10^4$
5^{-3}	ND	ND	$9.6x10^3$
5^{-4}	ND	ND	$3.4x10^3$
NTC & NEC	ND	ND	ND

NTC – no template control. NEC – negative extraction control. ND – not detected.

4.2.3 Validation of HIV-1 DNA-load Assay on Patients Infected with Varying HIV-1 Subtypes

Ten samples of differing HIV-1 subtypes were selected, the buffy coat layer removed and DNA extracted using the EZ-1 extraction procedure. Samples were tested as described previously using the HIV-1 LTR, β-globin, PhHV qPCRs.

HIV-1 LTR copies/10^6 PBMC were calculated using the following formula (there are 2 LTR copies per HIV-1 virus, and also 2 β-globin copies in every human cell) :

$$\text{HIV-1 DNA copies/}10^6\text{ PBMC} = \frac{\text{LTR copies}}{\text{β-globin copies}} \times 10^6$$

$CD4^+$ T-cell percentage was obtained from routine laboratory data available on the day (or near to the day) the sample was taken which was subsequently used for viral DNA-load assessment. Since this value gives what percentage of lymphocytes are $CD4^+$ it was used to estimate HIV-1 LTR copies /10^6 $CD4^+$ T-cells (since the majority of PBMCs are lymphocytes) using this formula:

$$\text{HIV-1 DNA copies /}10^6\text{ CD4}^+\text{ T-cells} = \frac{\text{HIV-1 LTR copies/}10^6\text{ PBMC}}{\%\ \text{CD4}^+\text{ T-cells}} \times 100$$

The experiment demonstrated amplification from all samples, with viral DNA-loads ranging from 10-545 copies/10^6 PBMC prior to conversion to copies/10^6 $CD4^+$ T-cells.

It was noted, however, the internal PhHV controls showed evidence of PCR inhibition (2.5 cycle right shift of the C_t). HIV-1 copy number would not be reliable in view of this. After several experiments designed to determine the potential cause of this it was found to be due to metal beads present in the extraction eluate which were used as part of the EZ1 biorobot extraction process. Centrifuging the eluate at high speed and applying a magnet to the tube in order to pipette off the sample without the metal beads resolved this inhibition problem.

4.2.4 Inter-Assay and Intra-Assay Reproducibility of HIV-1 LTR and β-globin qPCRs

Separate HIV-1 LTR and β-globin qPCR results were obtained for eight patient samples. As demonstrated by the duplicate copy number values for HIV-1 LTR in run 1 and 2 (Table 4.4), intra-assay variability was found to be minimal. Inter-assay variability was also minimal as shown by the values obtained for HIV-1 LTR copies / 10^6 PBMCs in each run for each sample. This demonstrates that the assay has good intra and inter assay reproducibility.

Table 4.4 Inter-assay and intra-assay variability for HIV-1 LTR and β-globin qPCRs.

	HIV-1 LTR copies				β-globin copies		HIV-1 LTR copies/10^6 PBMC	
	1		2		1	2	1	2
1	143.4	110.7	95.4	97.4	350236	422072	362.8	228.4
2	31.0	7.1	23.4	32.7	889404	813633	21.4	34.5
3	ND	ND	ND	ND	672812	530451	0	0
4	1.3	0.4	0.2	0.6	616095	485927	1.4	0.8
5	ND	ND	ND	ND	705341	476486	0	0
6	20.0	132.0	99.0	91.1	716092	860252	106.1	110.5
7	88.5	113.7	77.7	89.5	1064632	699953	95.0	119.4
8	31.0	41.0	22.9	15.3	953261	1297324	37.8	14.7

1 and 2 denote different qPCR runs, with duplicate aliquots tested on each run.
HIV-1 LTR copies/10^6 PBMC for each run (1 and 2) calculated using mean for LTR copies.
ND – not detected.

4.3 Discussion of Quantitative HIV-1 DNA-Load Assay

Previous studies have used various methods of reporting HIV-1 DNA-load in blood: copies/10^6 PBMC [313, 353, 355], copies / 10^6 $CD4^+$ T-cells [363, 364] or copies/µL of whole blood [365]. Most methods use Ficoll-Hypaque separation of whole blood to purify the PBMCs as described in the method here. We expected viral DNA-loads to be particularly low in viraemic controllers [314]. The advantages and disadvantages of the various ways of reporting HIV-1 DNA-load in blood are reviewed by Avettand-Fènoël *et al.* [366].

Reporting viral DNA-load as copies/ml of whole blood is susceptible to inconsistencies in the Ficoll-Hypaque separation procedure between samples, but may eliminate the influence of variation in cell counts in blood. The use of a parallel β-globin qPCR allowed normalisation of the HIV-1 DNA copies for 10^6 cells. Normalising the results minimises the influence of variations in the steps preceding quantification that are essentially linked to the yield of the DNA extraction step. Reporting viral DNA-load as copies/10^6 PBMCs means that the denominator cells include all those which might be HIV-1 target cells ($CD4^+$ T-cells and monocytes). Expressing results as copies/10^6 $CD4^+$ T-cells disregards monocytes, less often infected than circulating $CD4^+$ T-cells, but has the advantage of not being influenced by the variation in circulating $CD4^+$ T-cell pool (the main reservoir in blood). Given the fact that the purpose of this study was to compare two groups defined by their different $CD4^+$ T-cell counts, it was crucial that the results should not be open to bias introduced by differing $CD4^+$ T-cell counts; thus it was decided to report results expressed as copies/10^6 $CD4^+$ T-cells rather than copies/10^6 PBMCs. However, a potential problem with this approach is that all HIV-1 DNA is attributed to a $CD4^+$ T-cell reservoir, whereas in fact some may be harboured by circulating monocytes. Although this is typically a minor viral reservoir, in certain populations the amount of HIV-1 DNA in monocytes can be higher, such as in HIV-1 associated dementia [136]. Another issue with the method used is that copies/10^6 $CD4^+$ T-cells was estimated from copies/10^6 PBMCs using percentage $CD4^+$ T-cells; this is only an approximation since nucleated cells in PBMC suspensions are composed of lymphcotyes, monocytes and natural killer cells [367]. However, lymphocytes are the predominant cell type (around 85%) [367] and so this calculation should give a reasonable prediction of copies/10^6 $CD4^+$ T-cells. Whilst small errors may persist, since this calculation method was used for all samples it should still provide a reliable method for comparing viral DNA-load between the groups which is the main aim of the study. Another approach would have been to purify the $CD4^+$ T-cells from the PBMCs before DNA extraction. However, this could lead to a severe loss of cells and compromise sensitivity. In addition, the PBMC samples from many of the patients had been accumulated

over time and therefore were frozen, which is likely to have led to clumping on thawing thereby affecting the efficiency and reproducibility of $CD4^+$ T-cell sorting [368].

Taqman real-time qPCR allows simultaneous amplification and quantification, which eliminates the need for further manipulation of PCR products. This limits the risk of contamination. The qPCRs were thoroughly validated and optimised. Good specificity was ensured by the inclusion of multiple no template controls. Further specificity assurance was provided by using an assay which used a specific probe (unlike SYBR Green based amplicon). In terms of sensitivity of the HIV-1 LTR qPCR, the dynamic range of the assay was shown to be wide, covering at least five orders of magnitude (see Figure 4.1). The limit of detection of the HIV-1 LTR qPCR as suggested using molarity calculations was <1 copies per reaction. It was not possible to further define sensitivity since there is no agreed standard and there are no commercial assays for HIV-1 DNA-load quantification. The inclusion of an internal PCR inhibition control (PhHV) ensured inhibition was detected readily and the problem addressed. The assay demonstrated good intra (all samples had duplicate HIV-1 LTR qPCR on each run) and inter assay (several samples were repeated on different runs for verification) reproducibility. The exponential amplification phase of the qPCRs showed a strong linear relationship between the threshold cycles (C_t, number of amplification cycles required to reach a set threshold) and the $\log_{10}$ of the input copy number (r^2 value was consistently $\geq$0.99). The qPCRs were also quick and easy to perform, minimising the chances for error. Crucially in this setting the assay was both designed (choice of primers and probes) and validated for use on a wide range of HIV-1 subtypes including the various non-B subtypes encountered when testing samples from the East London HIV-1 population.

HIV-1 DNA–loads obtained here were compared with those in the literature. Using this assay viral DNA-loads ranged from 10-545 copies/10^6 PBMC (prior to conversion to copies/10^6 $CD4^+$ T-cells); this is comparable to values found by Rouzioux *et al.* in untreated HIV-1 infected subjects: 2.45 - 3.32, median 2.86 $\log_{10}$ copies/10^6 PBMCs (which is 282 – 1622, median 724 copies/10^6 PBMCs). However, values obtained here were approximately a $\log_{10}$ lower than those reported by Gibellini *et al.*: 2063 ± 2144 (mean +/- 1SD), range 126-9736, copies/10^6 cells [369]. It is difficult to compare the results of these studies as different quantitative techniques were used and on differing populations.

In summary, this work describes a novel real-time qPCR that allows quantification of HIV-1 DNA-load over a wide dynamic range with good inter and intra assay reproducibility. This assay has excellent sensitivity and can be used for HIV-1 DNA-load quantification of a wide range of HIV-1 subtypes and has been validated for use on PBMC samples.

4.4 Methods

4.4.1 Summary Method for Quantitative HIV-1 DNA-load Assay

Samples were tested using the HIV-1 DNA-load assay from a range of discord controllers, typical controllers and non controllers. Non controllers were selected on the basis of being ART-naïve HIV-1 infected persons, infected >12 months with a viral RNA-load >10000 copies/ml and a range of $CD4^+$ T-cell counts (see Figure 2.1, non controllers are shown in green). Age, sex and routine laboratory results (see Section 2.2.2) were recorded for each patient. A geometric mean was calculated of the last three $CD4^+$ T-cell counts and HIV-1 RNA-loads prior to the date of the sample used for HIV-1 DNA-load was taken. Following on from assay development, here is the summarised protocol used for viraemic controller patient samples:

PBMCs had been previously cryopreserved from patient's blood, prepared by Ficoll-Hypaque separation of EDTA anticoagulated blood (see Appendix I). DNA was extracted from these cells after thawing using the EZ1 Qiagen biorobot and DNA Tissue programme according to the manufacturer's instructions into a 200µl eluate. A fixed concentration of PhHV was added to each sample before extraction as an internal amplification control. A final 25µl qPCR mixture contained 5µl of DNA extracted from PBMC, 2×PCR QuantiTect Multiplex RT-PCR No Rox Mix (Qiagen), 0.2µM of each HIV-1 LTR primer (F1 5'-AGCCTCAATAAAGCTTGCCTTGA-3'; R1 5'-GGCGCCACTGCTAGAGATTTT-3') and 0.2µM probe (AAGTAGTGTGTGCCCGTCTGT, fluorescent label). Thermocycling conditions (ABI 7500) were 95°C for 15 minutes; then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Duplicate HIV-1 LTR and β-globin qPCRs were run in parallel for each sample and mean value calculated. An external plasmid dilution series was run in order to construct a standard curve for both HIV-1 LTR and β-globin, so that copy number could be determined in the test samples. This data allowed viral DNA-loads to be expressed as per cell (PBMC) equivalent. This was run each time to allow for differing efficiencies of a particular qPCR run, thus allowing robust comparison of values obtained on different runs.

4.4.2 Statistical Analysis

Statistical analysis and graphical presentations were performed using Prism (version 4.0, Graphpad) and results were considered significant if $p<0.05$. To determine significant differences in viral DNA-loads between cohorts, a 2-tailed Mann-Whitney U test was applied. Spearman's test was used to determine correlation between viral DNA-load and contemporaneous $CD4^+$ T-cell count.

4.5 Results

4.5.1 Patient Demographics and Laboratory Parameters

Demographics together with $CD4^+$ T-cell count and HIV-1 RNA-load distributions in patient groups tested using the HIV-1 DNA load assay are shown in Table 4.5.

Table 4.5 Demographics, HIV-1 RNA-loads and $CD4^+$ T-cell counts in patients tested for HIV-1 DNA-load.

	Discord Controllers (n = 11)	Typical Controllers (n = 16)	Non Controllers (n = 10)
Age, years	38 (22 – 64)	42.5 (31 – 71)	42 (28 – 66)
Female	63%	8%	10%
$CD4^+$ T-cells/mm^3	339 (251 – 424)	775 (499 – 1326)	399 (258 – 716)
HIV-1 RNA-load (copies/ml)	412 (49 – 1296)	391 (40-1745)	15,809 (10,041 – 169049)

Age, $CD4^+$ T-cell counts and HIV-1 RNA-loads are expressed as median (range).

4.5.2 HIV-1 DNA-load in Discord Controllers and Typical Controllers

Using the HIV-1 DNA-load assay developed in Section 4.2, viral DNA-loads were measured in discord controllers, typical controllers and non controllers. This generated a value for viral DNA copies/10^6 PBMCs for each patient. This was then used together with the $CD4^+$ T-cell percentage obtained from a contemporaneous result from the routine laboratory testing (see Section 2.2.2) to calculate a value for the viral DNA-load (copies/10^6 $CD4^+$ T-cells). The results are shown in Table 4.6.

Viral RNA-load (copies/ml) and viral DNA-load (copies/10^6 $CD4^+$ T-cell) for each cohort tested are shown in Figure 4.2. Viral RNA-load in the discord controller cohort is similar to that found in typical controllers (Figure 4.2 b). In contrast, the discord controllers have significantly higher levels of viral DNA-load than typical controllers; these levels were found to be comparable to those in non controllers (Figure 4.2 a).

Table 4.6. HIV-1 DNA-load /10^6 PBMCs, $CD4^+$ T-cell percentage and HIV-1 DNA-load /10^6 $CD4^+$ T-cells for each patient tested.

Patient	HIV-1 DNA-load copies / 10^6 PBMCs	$CD4^+$ T-cell % (of lymphocytes)	HIV-1 DNA-load copies / 10^6 $CD4^+$ T-cells
Discord Controllers			
1	1.33	10	13.27
2	70.32	4.6	1528.68
3	62.30	9.6	648.92
4	24.98	10.5	237.87
5	22.44	10.2	220.02
6	78.70	10.32	762.59
7	47.37	8.53	555.35
8	44.98	5.83	771.47
9	53.21	7.59	701.04
10	33.20	5.52	601.44
11	23.52	9.1	258.44
Typical Controllers			
1	2.74	9.2	29.74
2	7.80	7.5	103.99
3	0	19.2	0.00
4	1.91	4.0	47.83
5	33.26	11.7	284.26
6	14.04	8.0	175.50
7	31.26	14.3	219.28
8	25.02	11.2	223.43
9	0	16.8	0.00
10	0.33	8.4	3.90
11	19.82	14.2	203.26
12	14.79	11.5	128.53
13	80.72	10.7	755.25
14	2.12	10.2	20.77
15	7.99	11.4	69.86
16	1.21	19.7	6.16
Non Controllers			
1	110.43	12.6	876.45
2	78.15	10.5	744.25
3	97.91	6.9	1419.04
4	119.06	13.8	862.74
5	52.62	5.4	974.53
6	1.46	5.5	26.56
7	34.41	4.3	800.32
8	124.74	5.7	2188.41
9	131.66	11.1	1186.09
10	174.97	6.7	2611.52

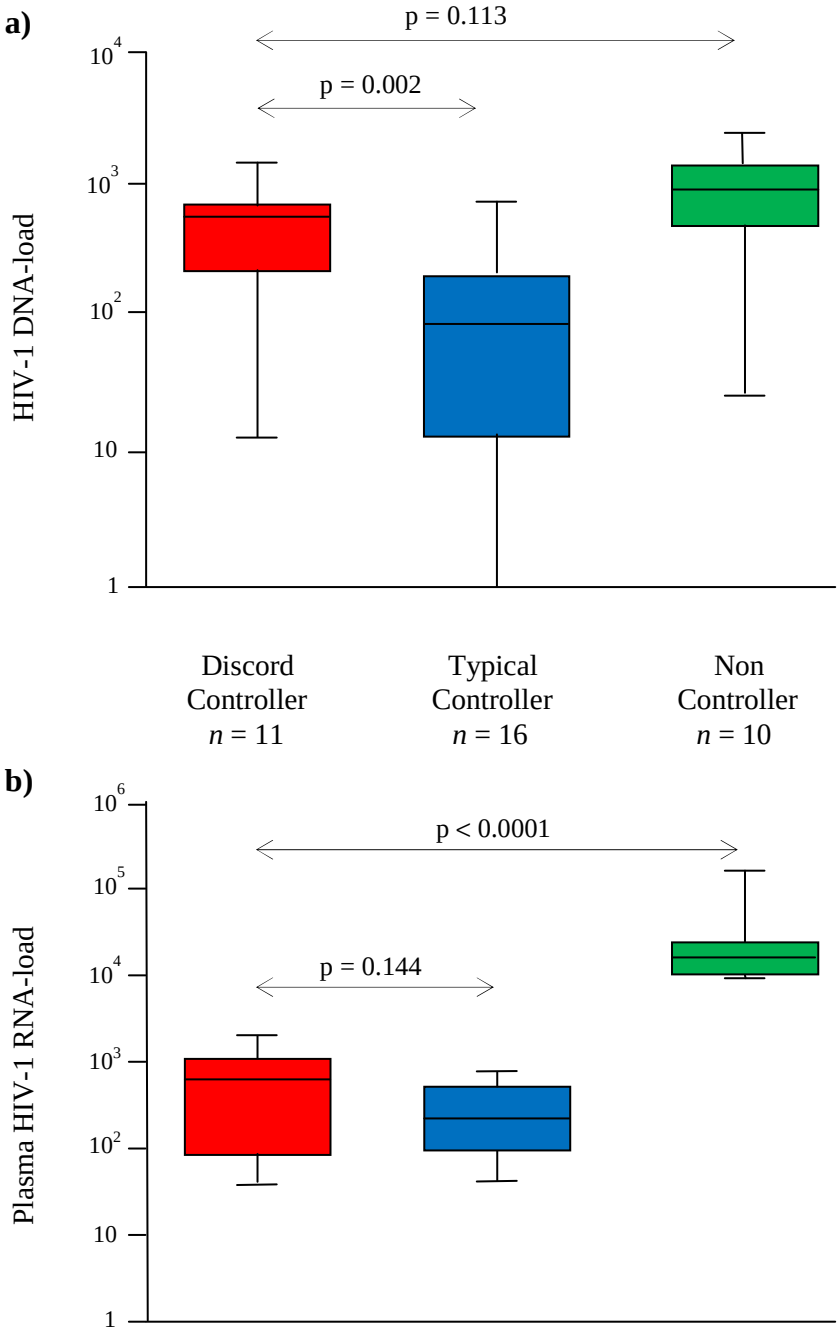


Figure 4.2 HIV-1 DNA-loads and RNA-loads in Discord Controllers, Typical Controllers and Non Controllers. HIV-1 RNA-load (copies/ml), a, and HIV-1 DNA-load (copies/10^6 $CD4^+$ T-cells), b, are both displayed on a $\log_{10}$ scale. Each central bar represents the median value, each box represents the IQR. Whiskers show the minimum and maximum values. Median (range) copies/10^6 $CD4^+$ T-cells for each group: typical controllers, 87 (0 - 755); discord controllers, 601 (13 – 1529); non controllers, 852 (27 – 2188).

Figure 4.3 shows $\log_{10}$ HIV-1 DNA-load plotted against mean $CD4^+$ T-cells/mm^3 for all viraemic controller patients (discord controllers and typical controllers). A strong inverse relationship between viral DNA-load and $CD4^+$ T-cell count was demonstrated.

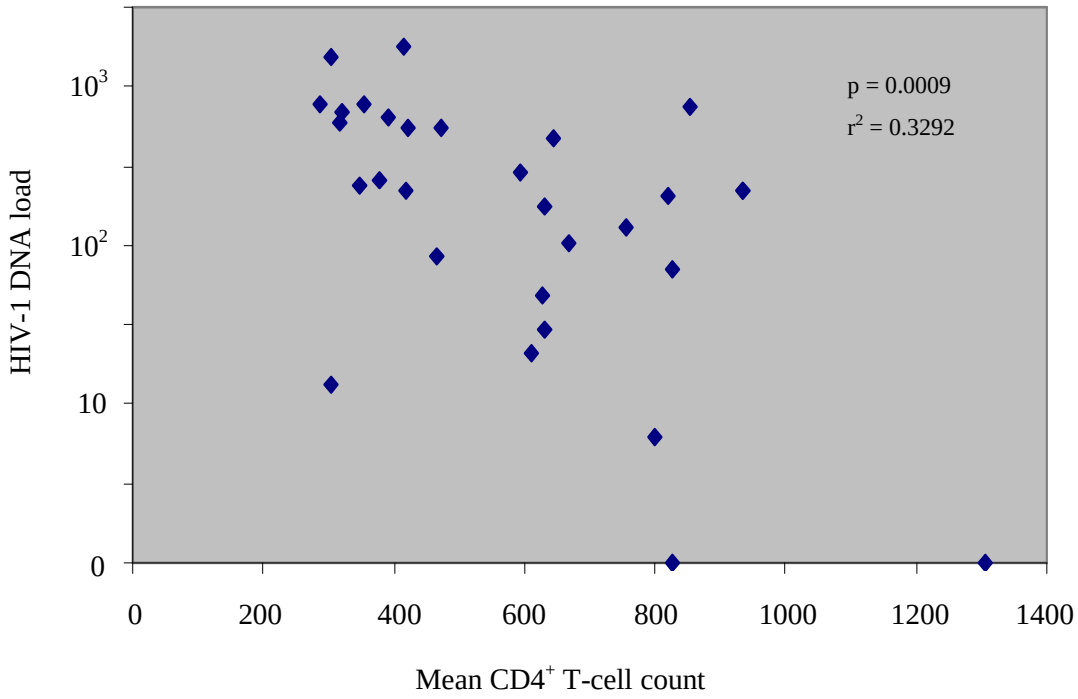


Figure 4.3 HIV-1 DNA-load Plotted Against Mean $CD4^+$ T-cells/mm^3 for all Viraemic Controllers. HIV-1 DNA-load (copies/10^6 $CD4^+$ T-cells) displayed on a $\log_{10}$ scale**.** Mean $CD4^+$ T-cell count is the geometric mean of the last three counts prior to the date of the sample used for HIV-1 DNA-load.

4.6 Discussion

Discord controllers have lower $CD4^+$ T-cell counts but equivalent HIV-1 RNA-loads compared with their typical controller counterparts; one explanation would be that discord controllers have higher levels of HIV-1 replication despite the fact this is not reflected in the viral RNA-load. In order to determine whether HIV-1 replication is higher in discord controllers than typical controllers, HIV-1 DNA-load was measured as a further marker of viral replication. HIV-1 DNA-load has been shown to be a marker of viral replication and disease progression. For example, it has been shown that the HIV-1 DNA-load in PBMCs from untreated patients provides an estimate of the cellular viral reservoir, which is established soon after infection [350, 370] and independently predicts disease progression [313, 350, 351]. HIV-1 DNA-load decreases over time in patients on ART [369]. Furthermore, higher HIV-1 DNA-loads are associated with suboptimal recovery of $CD4^+$ T-cell count in patients on ART [371, 372]. A negative correlation between viral DNA-load and $CD4^+$ T-cell count is also seen in HIV-2 infected patients, where viral RNA-load is often undetectable [373-375].

HIV-1 DNA-loads may be determined by measuring total cellular viral DNA, which includes both integrated provirus and unintegrated virus (unintegrated linear DNA, as well as 1-LTR and 2-LTR circles) (see Section 1.4). It could be argued that measuring integrated HIV-1 DNA-load would be a more appropriate measurement since it is from this integrated form that transcription leading to progeny virions occurs [88]. A method has been described for quantification of integrated HIV-1 DNA using an *Alu*-LTR PCR technique whereby one primer anneals to the HIV-1 LTR and a second in the highly repeated chromosomal *Alu* element [376]. However, integrated HIV-1 DNA appears remarkably stable, with one study showing that after 48 weeks ART a 5-fold reduction in the total HIV-1 DNA-load was seen without a parallel decrease in integrated viral DNA [352]. Since ART is well documented to lead to a decrease in viral replication, it follows that integrated HIV-1 DNA may not be an accurate marker of current replication. Further studies confirm that integrated HIV-1 DNA forms have a very long half-life [377] and as such are likely to represent the fundamental latent viral reservoir [86]. In contrast, unintegrated HIV-1 DNA, the more unstable form, has been proposed to be a good marker for ongoing replication *in vivo* [356]. Work from several groups has shown the persistence of 2-LTR circular forms in PBMCs of HIV-1 infected individuals in the absence of detectable viral RNA-loads, and has been correlated with residual cryptic viral replication [356, 378]. As such, many of the papers which have reported that HIV-1 DNA-load is a marker of progression or of high viral replication have employed assays measuring total cellular HIV-1 DNA-load (including the unstable unintegrated forms) [313, 350, 352, 353]. For this reason, an assay which determined total cellular HIV-1 DNA-load was developed.

Of note, the range of HIV-1 DNA loads obtained during this work were more narrow that was seen for HIV-1 RNA loads; this more restricted range of values for HIV-1 DNA loads has been reported previously [379].

Work described here found low HIV-1 DNA-loads in typical controllers: median 87 (range 0 – 755) copies/10^6 $CD4^+$ T-cells; when expressed as copies/10^6 PBMCs values are median 7.90, (range 0 - 80.72). This is in keeping with findings in other studies for example, Lambotte *et al.* found in elite controllers a mean total HIV-1 DNA-load of 32 (range 0 – 251) copies / 10^6 PBMCs [314]. Julg *et al.* found HIV-1 DNA-loads to be 12.79 ± 20.92 DNA copies/10^6 PBMCs in elite controllers and 94.80 ± 95.47 in viraemic controllers [380].

A literature search suggests that HIV-1 DNA-loads have not previously been reported in viraemic controllers with low $CD4^+$ T-cell counts, i.e. discord controllers. Work reported here shows that HIV-1 DNA-loads in discord controllers, median 601 (range 13 – 1529) copies/10^6 $CD4^+$ T-cells, are significantly higher than in typical controllers, and that they are equivalent to levels found in HIV-1 non controllers, median 852 (range 27 – 2188) copies/10^6 $CD4^+$ T-cells.

Thus it is possible to accept the hypothesis that there is higher HIV-1 replication in discord controllers compared with typical controllers as measured by HIV-1 DNA load, which is not apparent by observation of viral RNA-load alone.

In summary, using a qPCR to measure cellular HIV-1 DNA-load in blood, work here demonstrates that discord controllers with low $CD4^+$ T-cell counts (<450cells/mm^3) have significantly higher HIV-1 DNA-loads than typical controllers who have normal $CD4^+$ T-cell counts (>450cells/mm^3). HIV-1 DNA-loads in discord controllers are equivalent to those found in HIV-1 non controllers. An inverse correlation between HIV-1 DNA -load and $CD4^+$ T-cell count is seen in the viraemic controller cohort as a whole.

CHAPTER 5

Gut-Tropic $CD4^+$ T-cells in Discord Controllers and Typical Controllers, and the Relationship between HIV-1 DNA-load and subsequent HIV-1 RNA-load blips and $CD4^+$ T-cell decline

5.1 Introduction

It was reported in the last Chapter that cellular HIV-1 DNA-loads were high in discord controllers despite the lack of cell free plasma virus. This raises the possibility that viral replication may be compartmentalised. The GI tract, including both GALT (Peyer's patches and solitary lymphoid follicles) and lymphocyte effector sites (lamina propria and intraepithelial sites), has been proposed to be a major viral reservoir in HIV-1 disease [108, 381]. Study of SIV infection of rhesus macacques demonstrates that early on infected cells are seen in the lamina propria as well as in GALT, but that later on infected cells appear more restricted to GALT [382]. $CD4^+$ T-cells in the GI tract have been shown to be 10 times more frequently infected than those in peripheral blood in early HIV-1 infection, highlighting the potential for compartmentalised viral replication at this site [383]. Further evidence for such compartmentalised viral replication at this site comes from the finding that productively infected $CD4^+$ T-cells in GALT and the lamina propria are observed many years after the initiation of ART and long after suppression of viral RNA-load in the blood [384, 385]. In addition, distinct HIV-1 populations have been identified in the GI tract as a result of local genetic evolution, indicating that viral replication at this site may occur independently from the blood [386].

In order to study the possibility of compartmentalised viral replication in the GI tract most rigorously mucosal tissue samples would be taken to examine viral replication levels at this site in discord controllers and typical controllers. However, the invasiveness of such a procedure and the ethical constraints therefore surrounding this means such specimens are not commonly obtained. Consequently, two possible consequences of such compartmentalised viral replication in discord controllers were examined: evidence for enhanced migration of lymphocytes to the GI tract, potentially also accounting for depleted $CD4^+$ T-cells; and secondly the possibility that high viral DNA-load, and the compartmentalised viral replication it may represent, manifests itself as subsequent viral RNA-load blips. These two possibilities will now be discussed in more detail.

In HIV-1 infection the degree of inflammation within the GI tract correlates with viral replication levels [387, 388], this inflammation is likely to lead to migration of lymphocytes to this site. Migration of effector memory T-cells to intestinal mucosa and in the entry of naïve T-cells to GALT is facilitated by the interaction between integrin $\alpha4\beta_7$ on T-cells and mucosal addressin cell adhesion molecule (MAdCAM-1) in the vascular endothelium [389-392]. The $\alpha4\beta_7$ integrin is expressed on naïve T-cell in response to the vitamin A metabolite retinoic acid

[393]. In inflammatory bowel disease (IBD) and coeliac disease there is an enhanced level of lymphocyte migration to inflamed sites in the gut and consequently the level of T-cells in the blood expressing the gut homing integrin $\alpha4\beta_7$ are decreased [394, 395]. Furthermore, early during HIV-1 infection when viral replication and $CD4^+$ T-cell depletion in the GI tract is known to be very high, a significant depletion of blood $CD4^+$ T-cells expressing $\alpha4\beta_7$ integrin is also seen [396]. These $\alpha4\beta_7$ expressing $CD4^+$ T-cells are found abundantly in the GI tract and have been shown to be more susceptible to HIV-1 infection than cells not expressing the integin [397]. The integrin, which appears in a complex with CD4, may act in such a way to capture the virus thus enhancing infection rates since it has been shown to bind the HIV-1 envelope protein gp120 prior to engagement with CD4 and CCR5 [292, 398].

Therefore, the first specific hypothesis for the work described here was that inflammation arising from compartmentalised viral replication in the GI tract of discord controllers leads to increased T-cell migration to this site, which can be measured as depletion of $\alpha4\beta_7^+$ 'gut tropic' $CD4^+$ T-cells from the circulation in this group compared with typical controllers. It would follow that such enhanced migration of $\alpha4\beta_7^+$ $CD4^+$ T-cells to the GI tract would also result in enrichment of cells highly susceptible to infection at this site, thus enhancing infection even further. Therefore, an experiment was designed to determine whether there were fewer $\alpha4\beta_7^+$ $CD4^+$ T-cells in blood from discord controllers compared with typical controllers.

If viral replication is compartmentalised in discord controllers at a site such as the gut, the ongoing concealed replication might result in virions intermittently ‘spilling’ into the circulation. This would result in a spike or ‘blip’ in HIV-1 RNA-load. Indeed, higher HIV-1 DNA-loads in patients on ART have been shown to predict failure of treatment with viral RNA-load increases or blips [355, 399-401] thus suggesting the high viral DNA-loads represent high albeit concealed viral replication. Further to this, since HIV-1 DNA-load is a prognostic marker for disease progression in non controllers [313, 350, 351] it may be that this is also the case in viraemic controllers. Therefore, a second hypothesis was formulated whereby HIV-1 DNA-loads in viraemic controllers would predict subsequent HIV-1 RNA-load blips, $CD4^+$ T-cell decline and the need for ART initiation.

5.2 Methods

5.2.1 Identification of Gut-tropic T-cells using Flow Cytometry

The same patient groups were analysed as described in Chapter 3: discord controllers with a $CD4^+$ T-cell geometric mean of ≤400 cells/mm^3 and typical controllers with mean $CD4^+$ T-cells ≥500 cells/mm^3, avoiding those patients too near the 450 cells/mm^3 cut-off together with progressors and uninfected controllers. The method for staining T-cells and for flow cytometry is described in Section 3.2.1.

Since no $\alpha4\beta_7$ monoclonal antibody was available, a β_7-specific antibody was used as a surrogate. Whilst β_7 is also found to form the heterodimer receptor $\alpha E\beta_7$ (also known as $CD103\beta_7$) there are very few T-cell in the blood expressing this heterodimer; it is more often a characteristic of GI tract intra-epithelial lymphocytes facilitating retention of lymphocytes at this site via interactions with E-cadherin [392, 402]. Therefore, those T-cells in blood expressing β_7 can be assumed to be $\alpha4\beta_7^+$.

Central memory ($CD3^+CD45RO^+CD62L^+$) and effector memory ($CD3^+CD45RO^+CD62L^-$) $CD4^+$ and $CD8^+$ T-cells were stained with β_7–APC and appropriate isotype control in order to determine those expressing β_7. Data was again analysed using Winlist software in order to determine the percentage of cells expressing β_7 amongst each $CD4^+$ T-cell population.

In order to determine whether HIV-1 DNA-load predicts subsequently HIV-1 RNA increases or $CD4^+$ T-cell decline, subsequent routine laboratory values (see Section 2.2.2) were recorded for those patients in whom HIV-1 DNA-load was determined. For HIV-1 RNA the highest viral load over 1 year subsequent to HIV-1 DNA-load determination was recorded, together with the highest value obtained throughout all follow-up. $CD4^+$ T-cell count change from baseline (date of viral DNA-load) to the very next $CD4^+$ T-cell count (typically at 2-3 months), between baseline and last $CD4^+$ T-cell count obtained during the first year follow-up and between baseline and the last value obtained during follow-up were recorded. Which of these patients were subsequently started on ART was also recorded, although HIV-1 RNA-loads and $CD4^+$ T-cell counts whilst on treatment are not included in the follow-up analysis.

5.2.2 Statistical Analysis

Statistical analysis and graphical presentations were performed using Prism (version 4.0, Graphpad). Differences in percentage β_7 expression were assessed using a 2-tailed Mann-Whitney U test and results were considered significant if $p < 0.05$. A spearman test was used to determine correlation between HIV-1 DNA-load and subsequent HIV-1 RNA-load peaks and change in $CD4^+$ T-cell count.

5.3 Results

Patients analysed for all T-cell homing work were as described earlier in Table 3.1.

5.3.1 Percentage α4β7 Expression in CD4+ T-cells in Discord Controllers and Typical Controllers

As is shown in Figure 5.1, no difference in $\alpha4\beta_7$ expression in effector memory $CD4^+$ T-cells was observed between discord controllers and typical controllers. Neither were any significant differences in $\alpha4\beta_7$ expression observed in naïve or central memory $CD4^+$ T-cell subsets.

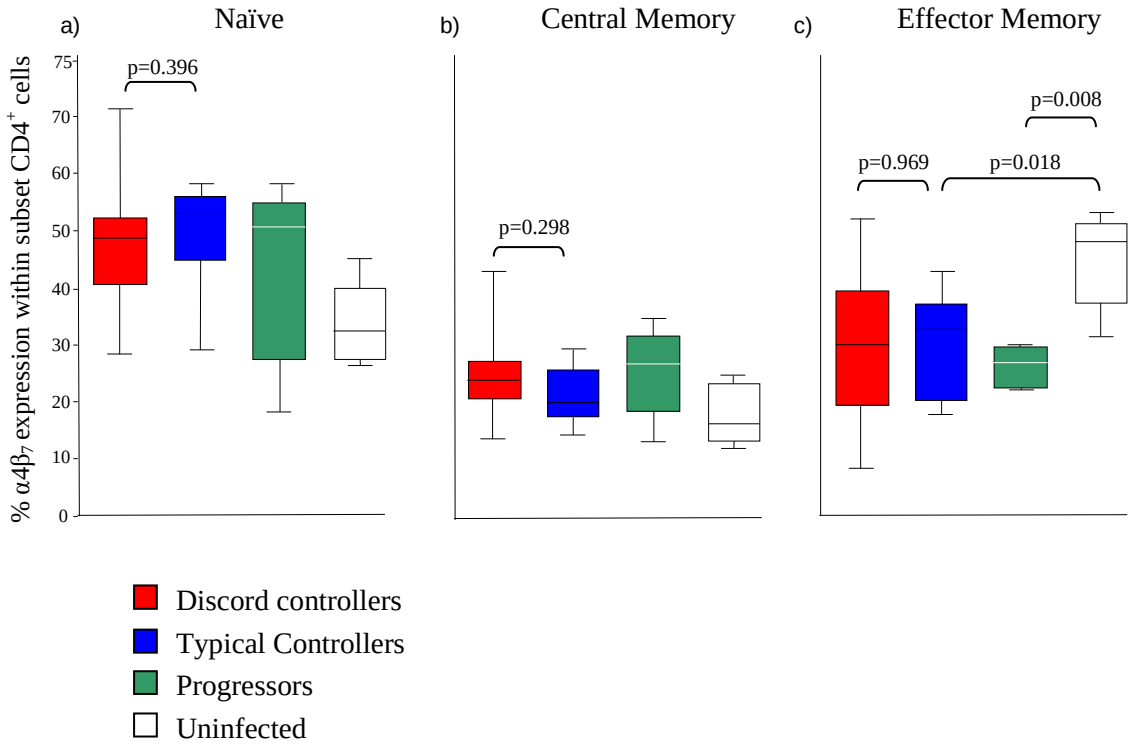


Figure 5.1 Percentage $\alpha4\beta_7$ expression in $CD4^+$ T-cells in Discord Controllers and Typical Controllers. Patients analysed included discord controllers and typical controllers, together with progressors and uninfected subjects for comparison. Percentage of $CD4^+$ T-cells expressing β_7 in a) naïve, b) central memory, and c) effector memory subsets. Each central bar represents the median value, each box represents the IQR and the whiskers represent the minimum and maximum values. Relevant p values are displayed.

5.3.2 Ability of HIV-1 DNA-load to Predict Change in HIV-1 RNA or CD4$^+$ T-cell in Viraemic Controllers.

HIV-1 DNA-load and subsequent HIV-1 RNA peaks and change in CD4$^+$ T-cell count in Viraemic Controllers is shown in Table 5.1.

No significant correlation was seen between HIV-1 DNA-load with either subsequent HIV-1 RNA or subsequent CD4$^+$ T-cell change (Spearman tests). High HIV-1 DNA-load was not associated with subsequent loss of control of viral RNA-load. This was further exemplified by the loss of HIV-1 RNA-load control in patients with low HIV-1 DNA-load (patients 9 and 10). Also, HIV-1 RNA continued to be controlled in some patients with very high HIV-1 DNA-load (patients 18, 19, 23 and 26). Neither was high HIV-1 DNA-load associated with subsequent CD4$^+$ T-cell loss. For example, patient 10 showed progressive loss despite low level HIV-1 DNA-load, yet others, such as patient 19, experienced a significant CD4$^+$ T-cell gain by the end of follow-up despite high level HIV-1 DNA-load. In line with this, high HIV-1 DNA-load was not associated with the subsequent initiation of ART. Patients 10, 18, 20, 24, 26 and 27 were started on ART, with many with equivalently high HIV-1 DNA-loads (13, 19, 22, 23, 25) not requiring ART during the follow-up period.

Table 5.1 Viraemic Controller HIV-1 DNA-loads and subsequent HIV-1 RNA-load peaks and $CD4^+$ T-cell change.

Patient	HIV-1 DNA-load (copies/10^6 $CD4^+$ T-cells)	HIV-1 RNA highest value 1 year (copies/ml)	HIV-1 RNA highest value all follow-up (copies/ml)	$CD4^+$ change at next test (cells/mm^3)	$CD4^+$ change 1 year (cells/mm^3)	$CD4^+$ change all follow-up (cells/mm^3)
Typical Controllers						
1	29.74	NDA	NDA	NDA	NDA	NDA
2	103.99	697	1118	-28	-86	-168
3	0.00	161	161	-368	-368	-118
4	47.83	<40	<40	-187	-75	-4
5	284.26	1403	1403	+125	+125	-41
6	175.50	NDA	17213	NDA	NDA	-209
7	219.28	443	39669	+192	+156	-224
8	223.43	135	10069	-313	-313	-661
9	0.00	2973	102902	+193	+193	-254
10	3.90	1643	19837	-106	-82	-246
11	203.26	NDA	2318	+108	-105	-105
12	128.53	NDA	NDA	NDA	NDA	NDA
13	755.25	NDA	NDA	-140	-140	-188
14	20.77	NDA	NDA	-127	-138	-138
15	69.86	NDA	435	NDA	NDA	-235
16	6.16	117	6166	+84	+259	+208
Discord Controllers						
17	13.27	125	991	-6	-86	-66
18	1528.68	240	240	-20	-20	-20
19	648.92	NDA	825	NDA	NDA	+170
20	237.87	1132	1320	+7	+11	-141
21	220.02	NDA	NDA	NDA	NDA	NDA
22	762.59	14584	28614	NDA	NDA	NDA
23	555.35	986	1160	+39	+49	-34
24	771.47	85	18755	-25	0	0
25	701.04	3015	281642	+31	-20	-41
26	601.44	NDA	273	+40	-34	-147
27	258.44	46	286	+55	-47	-214

NDA – no data available.

5.4 Discussion

The majority of $CD4^+$ T-cells lost during early HIV-1 infection are from sites within the GI tract [108, 118-120]. HIV-1 infection of $CD4^+$ T-cells at this site appears key to this dramatic loss since there is a preferential depletion of $CCR5^+CD4^+$ T-cells (i.e. those cells bearing HIV-1 receptors) which are present in high numbers in the GI tract lamina propria [121, 122]. This feature, as well as evidence of enhanced or independent viral replication [383, 386] and the presence of immune activation at this site despite successful treatment with ART [383], suggests that the GI tract may present a unique immunological environment which is particularly permissive to HIV-1 replication. This has led to the hypothesis that HIV-1 may 'hide' at this site, for example during ART [385, 403]. It is therefore also plausible that the virus might 'hide' from the immune system at this site in discord controllers in whom viral RNA-load levels are also suppressed, whilst destruction of $CD4^+$ T-cells at this site continues.

Migration of lymphocytes to GALT and to diffuse effector sites in the epithelium and lamina propria is mediated by a cell surface marker called integrin $\alpha_4\beta_7$ [292, 404]. This integrin is present on the cell surface of a small population of circulating T-cells. The major ligand for $\alpha_4\beta_7$, MAdCAM-1 [389], is selectively expressed on the endothelium of the intestinal vasculature [405] and is present in increased concentrations in inflamed tissue from patients with Crohn's disease and ulcerative colitis [406, 407]. Antibody-mediated inhibition of integrin $\alpha_4\beta_7$ reduces inflammation in animal models of colitis [408], and integrin β_7 knockout mice have small Peyer patches and have lower densities of lamina propria $CD4^+$ T-cells [409].

Studies on patients with Crohn's disease and ulcerative colitis, which are characterised by inflamed bowel mucosa, show that the proportion of systemic gut homing T-cells (expressing $\alpha4\beta_7$) are decreased compared with controls [395]. A further study found that in patients with active coeliac disease MAdCAM-1 expression in duodenal biopsies was significantly higher than in normal mucosa, and that percentage of T-cells in blood expressing β_7-positive was significantly lower in untreated coeliac disease compared with controls, but normalised after a gluten-free diet [394]. This led to the hypothesis that there is enhanced migration to the GI tract of $CD4^+$ T-cells in discord controllers compared with typical controllers accounting for their lower peripheral $CD4^+$ T-cell counts; if this were the case it could be expected that a lower proportion of circulating $CD4^+$ T-cells would express $\alpha4\beta_7$ in discord controllers.

The GI mucosa in HIV-1 infected patients is typically inflamed [384] and so lymphocyte recruitment to this site would be likely to be enhanced. In keeping with this, all HIV-1 infected subjects demonstrated a trend towards lower percentage $\alpha_4\beta_7$ expression in effector memory

$CD4^+$ T-cells compared with uninfected controls. Therefore, the data presented here supports the concept underlying this work that differences in gut inflammation due to viral replication at this site are reflected in the level of gut-tropic T-cells in blood. In contrast to the results presented here, Mavinger *et al.* have recently demonstrated an excess of circulating $CD4^+$ T-cells expressing $\alpha_4\beta_7$ in HIV-1 positive individuals compared with uninfected controls [410]. They hypothesise that this is due to defective homing leading to accumulation of T-cells expressing $\alpha_4\beta_7$ in the blood. They further hypothesise that defective homing leads to inadequate gut mucosal immune defence leading to increased microbial translocation and therefore increased T-cell activation. It might appear that these results conflict with data shown here, however, Mavinger *et al.* did not differentiate the different T-cell subsets expressing $\alpha4\beta_7$ and so it is possible that this excess in HIV-1 positive subjects were naïve T-cells. Further studies are needed to define lymphocyte trafficking in HIV-1 infection and whether HIV-1 infection leads to accumulation or depletion of circulating gut-tropic $CD4^+$ T-cells.

However, results presented here do not support the hypothesis that there is enhanced $CD4^+$ T-cell migration to the GI tract in discord controllers, since the percentage of peripheral $CD4^+$ T-cells expressing $\alpha_4\beta_7$ were found to be equivalent comparing discord controllers with typical controllers. Thus these experiments demonstrate no evidence of enhanced recruitment to the GI tract in discord controllers compared with typical controllers.

Of note is there high percentage of naïve T-cells which express $\alpha_4\beta_7$, since if these cells have yet to encounter antigen they would not be expected to expressing a homing marker. This is a curious result, but might be at least in part explained by the fact that some of the CD45RA expressing T-cells may in fact be effector T-cells in which CD45RO has reverted to CD45RA.

Another possible feature of compartmentalised viral replication is that it could give rise to either subsequent loss of control of viral RNA-load, if replication ceased to be restricted to the compartment, or disease progression as demonstrated by circulating $CD4^+$ T-cell loss. Since high HIV-1 DNA-load in viraemic controllers might be due to such compartmentalised replication it might predict loss of control of viral RNA-load or $CD4^+$ T-cell loss in these patients; this would be useful in the clinic to determine which viraemic controllers are most at risk of progressive disease and might therefore benefit from ART. Indeed, it has previously been reported that all three markers (viral RNA-load, $CD4^+$ T-cell count and viral DNA-load) should be combined to obtain a better picture of the dynamics of HIV-1 disease for each patient [313]. The data presented here on a small number of patients found no association between viral DNA-load and loss of RNA-load control or $CD4^+$ T-cell depletion in viraemic controllers. However, this study is limited and was not designed to determine this rigorously, since a small

group was studied and $CD4^+$ T-cell counts and HIV-1 RNA-loads were taken at inconsistent and irregular times during follow-up.

In conclusion to this Chapter, although there was evidence of reduced gut tropic effector memory $CD4^+$ T-cells in all HIV-1 infected groups there was no significant difference between discord controllers and typical controllers. Therefore, if there is an enhanced level of compartmentalised viral replication in the GI tract of discord controllers this does not impact T-cell homing to the GI tract that can be measured in blood. Furthermore, no evidence is found to suggest that HIV-1 DNA-load predicts HIV-1 RNA increase or $CD4^+$ T-cell decline during follow-up, which is a possible outcome of such compartmentalised viral replication.

CHAPTER 6

General Discussion and Future Work

6.1 Introduction

Viraemic controllers are rare patients with the capacity to control HIV-1 RNA-load in the absence of therapy. Whilst the reasons for this control are not completely understood, there is irrefutable evidence that untreated individuals with low viral RNA-load tend to demonstrate slower rates of $CD4^+$ T-cell decline in the blood and clinical progression than those with high viral RNA-load [304, 315, 316]. However, viral RNA-load by itself does not fully explain the variability in the rate of $CD4^+$ T-cell decline [259, 260]. Indeed, it has been reported previously that maintenance of a low viral RNA-load does not confer absolute protection against disease progression; for example, an individual in the Sydney Blood Bank Cohort who was infected with a partially attenuated HIV-1 isolate eventually developed immune deficiency despite a persistently low viral RNA-load [411]. Furthermore, $CD4^+$ T-cell declines are seen in HIV-2-infected individuals despite very low or undetectable viraemia [265].

In line with this, several patients were observed within the East London HIV-1 infected population with low viral RNA-load and counter intuitively they had low $CD4^+$ T-cell counts. The work described in this thesis developed from this observation and the clinical uncertainty that often accompanies these patients. Work here aims to study those patients with controlled viral RNA-load, but who demonstrated low $CD4^+$ T-cell counts and hence progression to AIDS, termed here 'discord controllers'. Specifically, the aims were to determine whether this cohort differed from typical controllers with maintained $CD4^+$ T-cell counts, in terms of epidemiological factors, virological characteristics or immunological factors. We also hypothesised that analysis of discord controllers with comparison to typical controllers would allow immunological changes associated with disease progression and those due to high viral RNA-load to be distinguished; this is not possible with analysis of patients with the typical pattern of progression (high viral RNA-load, low $CD4^+$ T-cells).

Very few papers describe such 'discord controllers' despite the considerable uncertainly in the clinic since markers of progression are discordant. A few such papers describe these patients in terms of routine laboratory parameters [316-319], whereas others looked at a limited number of T-cell characteristics of these patients in an attempt to understand why there is disease progression [320, 321, 338, 346, 347] and have been discussed in light of the data presented in Chapter 3, which presents a comprehensive assessment of T-cell populations in these patients. The work described here involves a cohort of 18 discord controllers, which is larger than those previously described. None of the papers already published examined epidemiological features or viral subtype of the discord controller cohort compared with typical controllers, which is of

interest in terms of understanding genetic or viral features which might predispose to this disease phenotype. No study examined HIV-1 DNA-load in these patients which is of interest in terms of understanding if the phenotype is driven by viral replication. Only one team of investigators (not published at the time of starting this work) have described the response to ART in discord controllers [318, 322]; determining this response is of interest both clinically and in terms of understanding the mechanism of pathology in this cohort.

6.2 Epidemiological Features of Discord Controllers and Typical Controllers

Since none of these previous studies described epidemiological characteristics of discord controllers, it was important to determine whether any factor was over-represented in this group. However, median age, sex distribution, ethnicity, region of birth and risk behaviour were all found to be similar comparing the two controller groups.

It is important to note that there is variation in lymphocyte reference ranges comparing people of different ethnicities, and that there have been reports of lower $CD4^+$ T-cell counts in people of Afrocarribean background [333]. However, it seems unlikely that this has confounded the results since no difference in ethnicity was found when comparing discord controllers and typical controllers. Other data collected during the course of this work confirm this notion since discord controllers were found to have higher HIV-1 DNA-loads and a distinct T-cell subset distribution compared with typical controllers. Furthermore, of the five patients observed on ART three had significant increase in $CD4^+$ T-cell count after starting ART, suggesting inhibition of replication had stalled $CD4^+$ T-cell loss. Similar findings are also described in the recent paper by Okulicz *et al.* who show that $CD4^+$ T-cell gains in viraemic controllers starting ART were only slightly lower than those observed in non controllers. Gains were found to be more significant in those with $CD4^+$ T-cell counts of <500 cells/mm^3 than in those with $CD4^+$ T-cell ≥ 500 cells/mm^3 at ART initiation [322]. The assertion that the low $CD4^+$ T-cell counts in discord controllers is not merely a variation in $CD4^+$ T-cell nadir is also further supported by the fact that percentage $CD4^+$ T-cells was significantly lower in discord controllers compared with typical controllers (suggesting that there was a more marked absence of $CD4^+$ T-cells in comparison with other lymphocytes subsets in the discord controllers). The suggestion is also strengthened by the finding of significantly higher HIV-1 DNA-loads in the discord controllers compared with typical controllers, as will be discussed further in Section 6.6.

A crucial question was whether discord controllers are in fact a continuum of the typical controllers resulting from $CD4^+$ T-cell decline over time and as such have been infected for longer. It is not often possible to obtain data regarding date of seroconversion, so time since

first positive HIV-1 test was recorded as a surrogate for this. There is no significant difference comparing the two controller cohorts, suggesting the discord controllers are a distinct phenotype. The fact that age distribution is similar comparing cohorts also supports this.

6.3 HIV-1 Subtypes in Discord Controllers and Typical Controllers

Given the fact that some HIV-1 clades appear to behave differently in terms of transmission and pathogenicity, for example clade D may be more pathogenic [32], it was logical to argue that a specific HIV-1 clade might predispose to the discord phenotype. There was a trend towards increased frequency of subtype C in the discord cohort compared with typical controllers, but there are no reports of increased pathogenicity of this subtype. Larger studies would be needed to determine whether there is a true association between subtype C HIV-1 and the discord controller phenotype. If confirmed, a theoretical possibility explaining this association might be that subtype C virus leads to significant levels of compartmentalised replication and as such viral RNA-load might not be an accurate reflection of HIV-1 replication levels.

6.4 Summary of HIV-1 RNA-loads, HIV-1 DNA-loads and T-cell Characteristics in Discord Controllers and Typical Controllers

A summary of HIV-1 RNA-loads, HIV-1 DNA-loads and T-cell Characteristics in discord controllers and typical controllers is shown in Table 6.1.

These results show that the $CD4^+$ T-cell compartment in discord controllers differ from that in typical controllers and is indistinguishable from that seen in progressors. Both discord controllers and progressors exhibit low total peripheral $CD4^+$ T-cell counts, high HIV-1 DNA-loads, depleted naïve $CD4^+$ T-cell and increased $CD4^+$ T-cell activation. In contrast, when observing the $CD8^+$ T-cell compartment discord controllers do not look like progressors ($CD8^+$ T-cell subset distribution and activation levels) and are indistinguishable from typical controllers.

Table 6.1. Summary of HIV-1 RNA-loads, HIV-1 DNA-loads and T-cell characteristics in Discord Controllers and Typical Controllers.

	Discord Controllers	Typical Controllers	Progressors	Uninfected
HIV-1 RNA-load	Low	Low	High	Negative
HIV-1 DNA-load	High	Low	High	Negative
$CD4^+$T-cell counts	Low	Normal	Low	Normal
Naive $CD4^+$ T-cells	Low	Normal	Low	Normal
$CD4^+$ T-cell % Activation	High	Normal	High	Normal
Naïve $CD8^+$ T-cells	Normal	Normal	Low	Normal
Effector $CD8^+$ T-cells	Normal	Normal	High	Normal
$CD8^+$ T-cell % Activation	Low	Low	High	Normal
Effector $CD4^+$ T-cell % $\alpha4\beta_7$	High	High	Highest	Normal

6.5 Why do $CD4^+$ T-cells Decline Despite HIV-1 RNA-load Control in Discord Controllers?

The observation that elevated $CD4^+$ T-cell activation is common to both progressors and discords suggests that it may be the main mechanism leading to $CD4^+$ T-cell decline in both groups. Since data was obtained at a single time point only, it is not possible to determine using only the data obtained here whether the $CD4^+$ T-cell activation is a cause or consequence of $CD4^+$ T-cell depletion; it could be argued, for example, that CD4 T-cell depletion might lead to activation as a homeostatic response to low numbers. However, the fact that numerous studies have demonstrated that T-cell activation predicts disease progression [263-265, 267, 339-341] and conversely that initiation of ART leads to a rapid decline in T-cell activation which is followed by $CD4^+$ T-cell count restoration [120], means that it is reasonable to assume that in discord controllers, like progressors, $CD4^+$ T-cell activation is a driving factor in $CD4^+$ T-cell depletion. As well as leading to clonal exhaustion and activation-induced cell death [203], immune activation is thought to accelerate progression by constantly generating new $CD4^+$ T-cell targets since HIV-1 favours infection of activated $CD4^+$ T-cells [112].

The fact that $CD4^+$ T-cell activation occurs in discord controllers without the presence of significant viral RNA-load might seem unexpected. Whilst many patients with significant T-cell activation and consequent disease progression disease do have high viral RNA-loads, the

uncoupling of activation and viral RNA-load has been described previously: in SIV infection of sooty mangabeys (the natural host) viral RNA-load is high and yet T-cell activation is low [412]; furthermore, in a study of ART naïve children, percentage $CD4^+$ T-cell activation was seen to be associated with viral DNA-load and not with viral RNA-load [413] similar to the findings here in discord controllers.

Lower numbers and percentages of naïve $CD4^+$ T-cells were observed in both discord controllers and progressors compared with typical controllers and uninfected subjects. Therefore, a deficiency in $CD4^+$ T-cell regeneration capacity may be a contributing factor as suggested by other studies [295]. The decreased naïve $CD4^+$ T-cell numbers might also be related to the increased activation level of these cells in discord controllers, since it known that activation can drive these cells into an effector phenotype [414].

Another possibility is that circulating $CD4^+$ T-cells are sequestrated to another site. Activation has also been shown to induce display of adhesion and chemoattractant receptors which lead to sequestration of T-cells to exit the lymphoid circulation to non-lymphoid sites [415]. Therefore, a model could be proposed whereby immune activation not only drives peripheral $CD4^+$ T-cell depletion through direct cytopathic effect, clonal exhaustion and apoptosis, but also through driving naïve cells into an effector phenotype which then exit to a non-lymphoid site. Whilst an increase in the effector memory phenotype was not seen in discord controllers compared with typical controllers (just a reciprocal increase in percentage due to naïve $CD4^+$ T-cell loss), only the blood compartment was studied, so the possibility that naïve cells have been driven into this phenotype and moved out of the circulation to their effector site cannot be excluded.

Based on findings that the GI tract is a major site of HIV-1 replication and $CD4^+$ T-cells depletion [113, 120], work described here investigated the possibility of enhanced sequestration to this site in discord controllers. The percentage of $\alpha_4\beta_7$ expressing effector memory $CD4^+$ T-cells tended to be lower in HIV-1 positive subjects compared with uninfected subjects, suggesting enhanced migration of these cells to the GI tract in all infected groups due to GI inflammation and/or viral replication at this site. This was not more marked in discord controllers compared with typical controllers, suggesting no evidence of enhanced sequestration to the GI tract in discord controllers compared with typical controllers.

6.6 Why Doesn't High HIV-1 DNA-load Give Rise to High HIV-1 RNA-load in Discord Controllers?

Transcription from integrated HIV-1 DNA gives rise to progeny HIV-1 virions and HIV-1 DNA integration results from infection of susceptible cells [88]. Therefore it is not clear why high HIV-1 DNA-loads seen in discord controllers, equivalent to those in non controllers, does not result in higher viral RNA-load. It could be argued that the high HIV-1 DNA-load in discord controllers mostly represents provirus-containing cells in latently infected resting memory cells rather than virus-producing cells. However, this seems an unlikely scenario based on several observations regarding total HIV-1 DNA (including unintegrated and integrated forms): total HIV-1 DNA-load decreases in patients put on ART as replication is suppressed [352, 353]; high total HIV-1 DNA load in patients on ART predicts viral RNA-load blips which suggests that higher viral DNA-load represents residual replication [355, 399-401]; total HIV-1 DNA-load predicts disease progression independently of viral RNA-load [313, 350]. Therefore, it seems likely that higher viral DNA-loads in discord controllers represent high viral replication level, particularly in view of their depleted circulating $CD4^+$ T-cell counts. This raises the important question of why this replication does not result in higher viral RNA-load in discord controllers.

It is a theoretical possibility that high level replication might lead to high HIV-1 DNA-load and yet low viral RNA-load if this replication is in large part compartmentalised. The central nervous system [416, 417] and the genital tract [418, 419] are well described candidate sites amongst others based on work demonstrating the generation of local viral variants or persistence of the virus during ART. The work described in Chapter 5 of this thesis focused on the possibility of compartmentalised replication in the GI tract based on observations suggesting enhanced or independent viral replication may occur at this site [383, 386] and because of the huge loss of $CD4^+$ T-cells at this site suggesting its particular importance in disease progression [120]. Whatever the site of such compartmentalisation, replication at this site could give rise to infected cells which contain HIV-1 DNA and re-enter the circulation (accounting for high viral DNA-load), and yet the free virus remains restricted to the site of replication (e.g. due to a physiological barrier such the blood-brain barrier) where it degrades either over time or as a result of the immune system.

Observation of the pattern of disease in HIV-2 infection might provide further clues to understand the uncoupled viral DNA- and RNA-load in discord controllers. For a given $CD4^+$ T-cell count viral DNA-load is similar when comparing HIV-1 with HIV-2 infection, although

viral RNA-load is much lower in HIV-2 infection [265]. The picture in HIV-2 infection therefore bears some resemblance to that seen in typical and discord controllers, where disease progression appears correlated with HIV-1 DNA-load but not viral RNA-load which remains low. In HIV-2 infection, virus-specific $CD8^+$ T cells maintain a polyfunctional profile similar to that in HIV-1 LTNPs [420]. Indeed, it could be that CTL mediated killing of productively infected cells might control viral RNA-load without having a significant effect on resting memory $CD4^+$ T-cells in which the HIV-1 provirus is integrated quiescently; hence HIV-1 DNA-load would be preserved in these long-lived cells and yet viral RNA-load would be low.

A further explanation that can be offered is that HIV-1 replication may be occurring in discord controllers in low-level bursts which may or may not give rise to transient viraemia. This transient burst of replication might therefore maintain and even increase numbers of infected cells over time, thereby leading to high cellular HIV-1 DNA loads and also the immune response to antigen, but only reflected in the viral RNA-load so transiently that it is easily missed by the infrequent laboratory tests. Such transient viraemia has been described in patients on ART and has been proposed to result from bursts of replication originating from infected resting memory cells [421-423]. A further possibility that could account for high HIV-1 DNA-load despite low RNA-load is that replication is predominantly occurring by cell to cell transmission as has been described previously [90]. It may also be that there is a defect in virion release from infected cells; intracellcular HIV-1 RNA levels would be expected in be elevated in discord controllers if this were the case.

On the other hand virions may be being produced effectively in discord controllers but cleared rapidly from the blood by humoral immune mechanisms. This may be due to enhanced efficiency of this arm of the immune system in discord controllers, or might be due to a virological feature. Indeed, in HIV-2 infection the virus has been shown to expose epitopes inducing nAbs in a way that does not occur in HIV-1 infection, which might explain the low plasma viral RNA-load in HIV-2 infection [424, 425]. These possibilities can be readily explored.

6.7 Why is the $CD8^+$ T-cell Compartment Different in Discord Controllers Compared with Progressors?

Whilst not a specific aim of this work, is it worth discussing why the $CD8^+$ T-cell compartment is markedly different comparing discord controllers with progressors, despite the fact $CD4^+$ T-cells depletion is a feature of both. Progressors were found to have a depleted naïve population, an expanded effector memory population and enhanced activation within the $CD8^+$ T-cell compartment. It is possible that the $CD8^+$ T-cell compartment changes seen in progressors may be a feature of high viral RNA-load rather than of disease progression. One explanation is that the high viral RNA-load might drive the proliferation of 'passenger' HIV-1 specific CTLs (those CTL which proliferate in response to viral antigen without themselves having effect on control of virus) [181]. This might account for the high numbers of effector memory $CD8^+$ T-cells in progressors, and is also supported by the finding that HIV-1 specific $CD8^+$ T-cell have been shown in decay following ART-mediated suppression of viral RNA-load [178]. Functional analysis or specificity of effector memory $CD8^+$ T-cells was not undertaken, but if it were it may show that these cells are specific for HIV-1 variants no longer present in the viral population, accounting for the fact viral RNA-load is uncontrolled in progressors. In terms of T-cell activation the findings described here are supported by a study by Giorgi *et al.*, who found higher viral RNA-load to be correlated with $CD8^+$ but not $CD4^+$ T-cell activation [264].

Whilst the $CD8^+$ T-cell compartment appears similar in the discord controllers and typical controllers it may be that there are qualitative differences of this compartment between controller groups. For example, the typical controllers may produce large numbers of HIV-1 specific 'driver' CTL which rapidly locate to tissues and control infection at these sites by targeting infected cells, hence reducing $CD4^+$ T-cell destruction. Functional studies would be needed in order to ascertain whether such qualitative differences exist.

6.8 What Implications Have These Findings Got for the Wider HIV-1 Infected Population?

This study enabled immunological changes associated with disease progression and those due to high viral RNA-load to be distinguished, and suggests that $CD4^+$ T-cell activation is not merely an effect of significant viral RNA-load, which itself leads to $CD4^+$ T-cell loss via some other mechanism. The observation that HIV-1 DNA-load is associated with $CD4^+$ T-cell activation and $CD4^+$ T-cell decline even in the absence of significant viral RNA-load supports a mechanistic link between viral replication (even if compartmentalised), chronic immune activation ($CD4^+$ compartment only) and progressive immunodeficiency.

The proposal that T-cell activation might be a stronger predictor than viral RNA-load of disease progression is most clearly supported by the fact that T-cell activation distinguishes pathogenic from non-pathogenic SIV infection in which viral RNA-loads are equivalent in each, but it is activation which is associated with progression in non-natural hosts (see Section 1.9.1) [412]. Silvestri *et al.* describe activation in the T-cell compartment as a whole in pathogenic SIV infection in non-natural hosts, but it appears that this activation is more sustained in $CD4^+$ T-cells than in $CD8^+$ T-cells. This might explain findings described here which suggest that $CD4^+$ T-cell activation may be more strongly associated with progression than $CD8^+$ T-cell activation. Further studies are required to delineate more specifically whether immune activation leading to disease progression affects both T-cell compartments or whether it is restricted to the $CD4^+$ T-cell compartment.

6.9 Future Work

One significant limitation of this study is its cross-sectional nature, since establishing cause and effect in such studies is difficult. Longitudinal studies on individual patients could examine differences in patients' immune systems and virus before and after loss of control of viral RNA-load control, or before and after $CD4^+$ T-cell depletion.

This thesis looked primarily at enumerating T-cells of a certain phenotype. However, evaluation of T-cell functional assays and effects on viral function in HIV-1 controllers would be helpful in elucidating further the mechanism of viral control. Further questions are yet to be answered here, including the specificity of the activated T-cells and whether this differs between controllers who do and don't maintain $CD4^+$ T-cell counts. Whole-genome association analysis, as has been described in viraemic controllers [305], may also be used to delineate specific features of the immune system in viraemic controllers which might predispose them to the discord controllers phenotype.

It would be of interest to determine whether the excess HIV-1 DNA levels in discord controllers compared with typical controllers represents unintegrated HIV-1 DNA as expected given its close relationship with viral replication [356]. It would also be useful to determine which $CD4^+$ T-cell population is the main reservoir for HIV-1 DNA in discord controllers, with resting memory $CD4^+$ T-cells [370] and macrophages [426] both potential reservoirs. Intracellular $CD4^+$ T-cell HIV-1 RNA levels could be measured in future studies in order to determine whether a defect in release of virus from infected cells might account for high HIV-1 DNA-loads and yet low RNA-load in discord controllers.

Further work on examining the potential factors driving excess immune activation seen in some HIV-1 infected groups including discord controllers could be undertaken. This would include HIV-1 antigens, non-HIV-1 antigens such as translocated microbial products, cytokines, stimulation through toll-like receptors or some combination of these or other factors. Several papers have determined levels of translocation from the gut by examining bacterial LPS levels in blood [320] or by employing bacterial ribosomal 16S quantitative DNA PCR [427]. Determining whether this might account for increased $CD4^+$ T-cell activation in discord controllers would help to determine why these patients experience disease progression.

The work presented here did not show that HIV-1 DNA-load predicts disease progression in viraemic controllers. However, a larger study group and sequential HIV-1 DNA-loads would be needed to examine this more thoroughly.

6.10 Conclusions

Study of the discord controller cohort provides a unique opportunity to understand the relationships between low level viraemia, adaptive immune responses and disease progression in HIV-1 infected individuals. This understanding could have a bearing on the development of therapeutic vaccine approaches and in understanding the mechanisms underpinning $CD4^+$ T-cell count decline.

The results presented in this thesis are consistent with a model whereby cellular HIV-1 DNA proviral load predicts naïve $CD4^+$ T-cell depletion and $CD4^+$ T-cell activation, whereas viral RNA-load is associated with naïve $CD8^+$ T-cell depletion, effector $CD8^+$ T-cell compartment expansion and $CD8^+$ T-cell activation.

How do these findings help clinicians? They remove some of the mystery surrounding why some patients with low viral RNA-load also have low $CD4^+$ T-cell counts. They confirm the relationship between ongoing viral replication and disease progression as shown by high HIV-1 DNA-load in discord controllers. In addition, they provide strong support for emerging efforts to manipulate the immune system, in particular those targeting immune activation and highlight the fact that immune activation levels could be a useful measure in assessing potential therapeutic vaccines.

APPENDICES

APPENDIX I

a) Recovery of PBMCs from EDTA Anti-coaglulated Blood using Ficoll-Hypaque Separation

1. Centrifuge to separate plasma and remove plasma into 50ml tube.
2. Dilute remaining blood with approximately equal volume phosphate buffered saline (PBS).
3. Add 15 ml Lymphoprep (Axis-Shield) to fresh 50ml tube.
4. Carefully layer 30 ml of the blood/PBS solution on top of the Lymphoprep (do not mix).
5. Centrifuge at 800 x g for 25 minutes at room temperature.
6. Pipette off the white 'buffy' layer between the two phases into a 50ml tube – these are PBMCs.
7. Fill up the tube with PBS to 50 ml.
8. Centrifuge at 800 x g for 5 minutes plus brake to pellet PBMCs.
9. Repeat wash step to obtain clear, clean looking pellet.
10. Resuspend pelleted PBMCs in 90% fetal calf serum (FCS) and 10% dimethyl sulfoxide (DMSO) and freeze slowly.
11. Store in liquid nitrogen.

b) Production of High Copy Number Plasmids with PCR Inserts for qPCR Standard Curves

1. The appropriately sized (bp) band was excised from the agarose gel with a scalpel.
2. Gel extraction and PCR purification using the QIAquick Gel Extraction Kit (Qiagen).
3. 'A-tailing' of purified PCR fragments -
    a. Template 10µl , 10x buffer 2µl ,*Taq* polymerase 0.16µl (2 U/µl) , dNTPs 0.2µl (1 mM final concentration).
    b. Incubate for 10 minutes at 72°C, then inactivate polymerase by freezing.
4. A-tailed PCR fragments were ligated into TOPO TA vector (Invitrogen) according to instructions for chemically competent *Escherichia coli*.
5. Transform Bacteria -
    a. 50µl of a heavy culture suspension of DH5α *Escherichia coli* was added to 2 µl TOPO ligation reaction.
    b. It was then heat shocked by putting the eppendorf in 42°C water bath for 30 seconds, following by moving to ice for 2 minutes.
    c. 200µl soc medium was then added, and incubated at 30°C for 30 minute.
6. Blue White screening -
    a. 50µl from each transformation was spread on agar plate containing ampicillin and incubated overnight at 37°C.
    b. Of the hundreds of colonies 10 white colonies were picked off into 45ml LB medium (with ampicillin) and incubated at 30°C overnight.
    c. This was then centrifuged to pellet the bacteria.
7. Purification of Plasmid DNA according to manufacturer's instructions for the QIAprep Spin Miniprep Kit.
8. In order to verify ligation of the PCR product a digest with eco R1 was performed -
    a. Buffer H 2µl, ecoR1 1µl, plasmid 5µl, water 12µl.
    b. Run on 2% agarose gel with ethidium bromide to check the appropriate sized insert.

c) Monoclonal Antibody Staining Matrix for Immunophenotpying using Flow Cytometry

Tube	FITC	PE	PerCP	PerCP-Cy5.5	PC7	APC	APC-H7	Pacific Blue
1	-	-	-	-	-	-	-	-
2	CD8	-	-	-	-	-	-	-
3	-	CD8	-	-	-	-	-	-
4	-	-	CD4	-	-	-	-	-
5	-	-	-	CD8	-	-	-	-
6	-	-	-	-	CD45RA	-	-	-
7	-	-	-	-	-	Beta7	-	-
8	-	-	-	-	-	-	CD4	-
9	-	-	-	-	-	-	-	CD3
-								
10	CLA	CD62L	HLA-DR	CD38	CD45RA	Beta7	CD4	CD3
11	CLA	CD62L	HLA-DR	CD38	CD45RO	Beta7	CD4	CD3
12	CCR4	CD62L	HLA-DR	CD38	CD45RA	Beta7	CD4	CD3
13	CCR4	CD62L	HLA-DR	CD38	CD45RO	Beta7	CD4	CD3
14	-	-	-	-	-	-	mIgG1	CD3
15	-	mIgG1	-	-	mIgG1	-	CD4	CD3
16	-	mIgG1	-	-	mIgG2a	-	CD4	CD3
17	rIgM	CD62L	mIgG2a	mIgG1	CD45RA	rIgG2a	CD4	CD3
18	mIgG2b	CD62L	mIgG2a	mIgG1	CD45RA	rIgG2a	CD4	CD3
19	rIgM	CD62L	mIgG2a	mIgG1	CD45RO	rIgG2a	CD4	CD3
20	mIgG2b	CD62L	mIgG2a	mIgG1	CD45RO	rIgG2a	CD4	CD3

Single colour tubes 1-9 are for colour compensation during analysis of data. Isotype controls are labelled as either mouse IgG (mIgG) or rat IgG (rIgG).

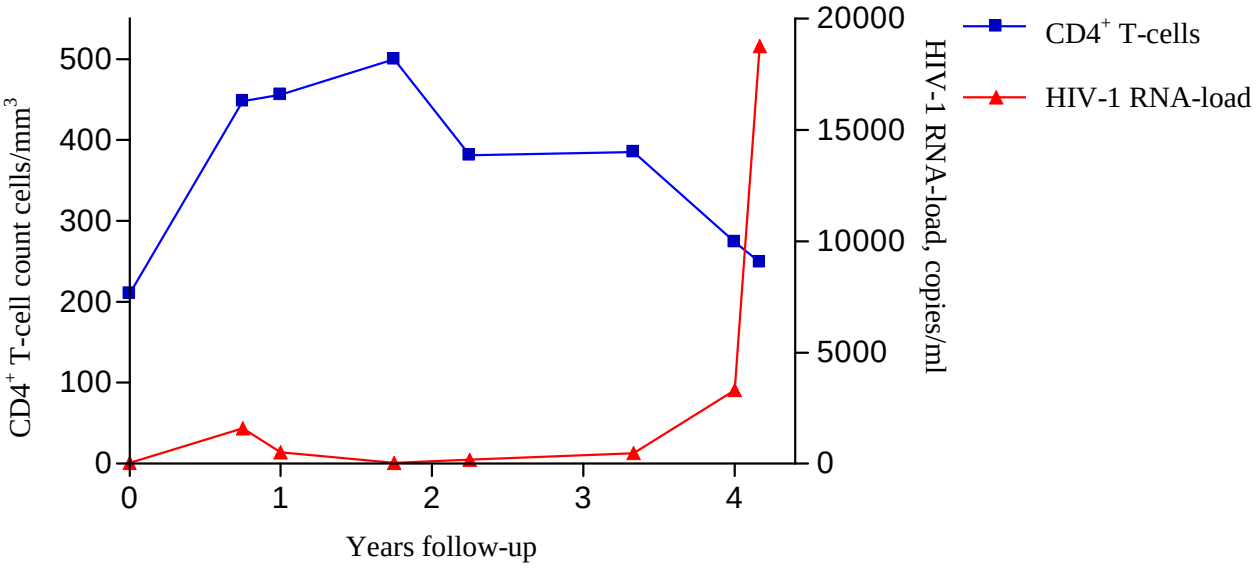
APPENDIX II

a) Loss of HIV-1 RNA-load Control in some HIV-1 Controllers

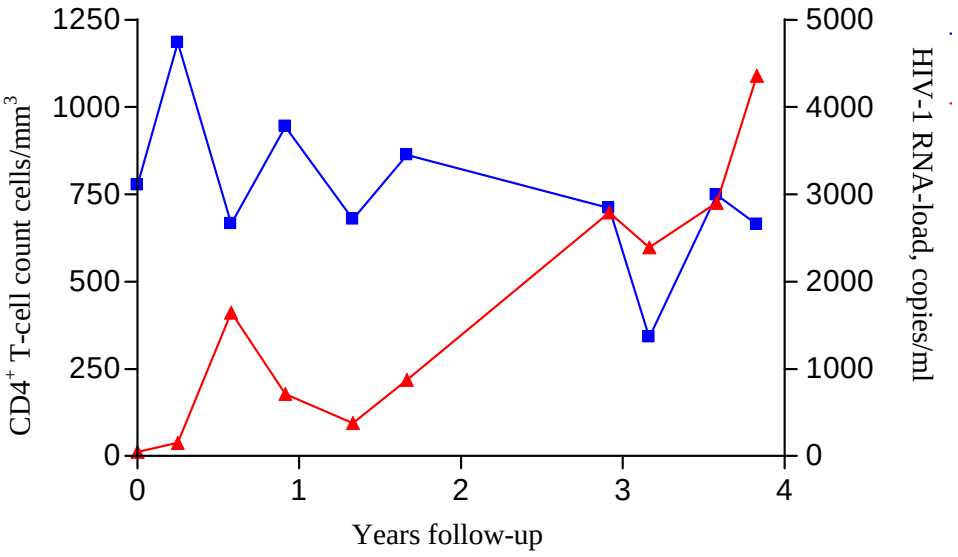
To describe this cohort fully, it was important to determine whether viral RNA-load and $CD4^+$ T-cell counts remained relatively stable for the majority of patients over time. This was particularly important when determining to which cohort the controller belonged. Whilst for most patients, viral RNA-load was stable, for a minority these values changed over time atypically (see Figure A.1).

- Loss of viral control 1: For one patient, over a period of over three years, viral RNA-load remained low with stable $CD4^+$ T-cell counts. After this viral RNA-load increased dramatically, with a concomitant fall in $CD4^+$ T-cell counts.

- Loss of viral control 2: In the typical-controller cohort two patients who fulfilled the controller inclusion criteria based on laboratory data available at our centre for 3.7 years (values prior to this not available; time since first positive test until loss of control of 6.4 years) and 3.5 years (i.e. for all values from the point of the first positive HIV-1 test), lost their controller status with a significant rise in viral RNA-load (latest values of 36411 and 4357 copies/ml) but maintained normal $CD4^+$ T-cell counts (732 and 708 cells/mm^3 respectively).



Loss of viral control 1: $CD4^+$ T-cell count drops concomitant with rise in HIV-1 RNA-load.



Loss of viral control 2: HIV-1 RNA-load increases whilst $CD4^+$ T-cell count is steady

Figure A.1 $CD4^+$ T-cell count and viral RNA-load over time for two patients in whom viral control was lost.

b) Incomplete HIV-1 RNA-load Measurements for Some Viraemic Controllers

For a minority of patients, classification was complicated by the fact that on occasion viral RNA-load was not requested during a clinic visit despite blood being tested for $CD4^+$ T-cell count. For example, one patient showed consistently low with normal $CD4^+$ T-cell counts for 2.3 years following first positive test (last viral RNA-load was 95 copies/ml at which point $CD4^+$ T-cell count was 510 cells/mm^3). However, viral RNA-load was not checked for a 10 month period following this, during which time $CD4^+$ T-cell counts dropped (310 & 326 cells/mm^3) prompting initiation of ART (see Figure A.2). This patient was classed as a typical controller since the $CD4^+$ T-cell count was > 450 cells/mm^3 at the last point viral RNA-load was known to be suppressed.

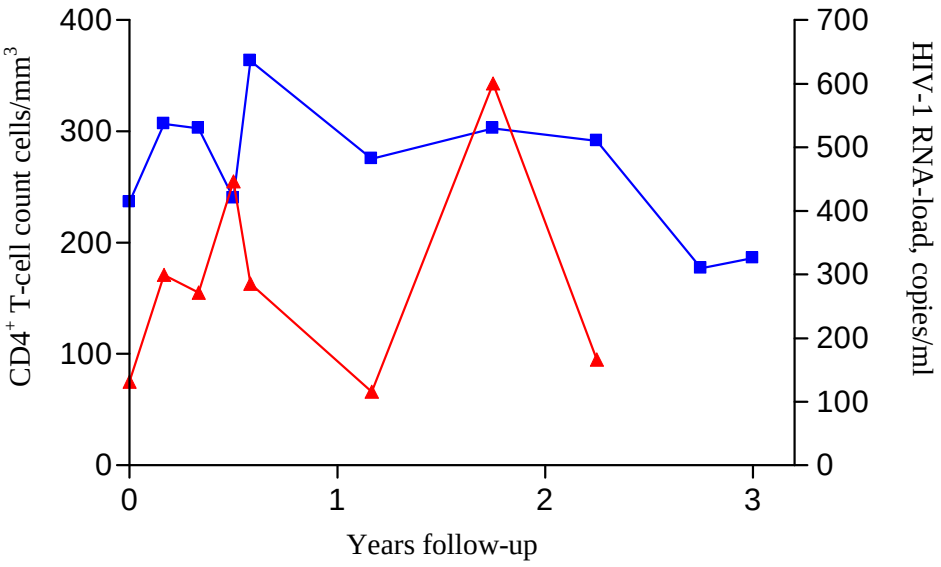


Figure A.2 Patient with incomplete values for HIV-1 RNA-load.

APPENDIX III

Table A.1 Co-morbid and Intercurrent illness for each Viraemic Controller

Patient	Clinical Condition
Typical Controllers	
1	Raynauds disease, seizure (unknown cause), depression
2	**Neurosyphilis, genital HSV infection**, spondylosis, gallstones
3	Nil
4	Nil
5	**Genital HSV**
6	**Hepatitis C virus infection**, asthma, hypogonadism
7	**Syphilis**
8	**Cervical intraepithelial neoplasia 2**, sickle cell trait
9	Nil
10	**Hepatitis C virus infection**
11	Depression
12	Nil
13	Nil
14	**Genital HSV**
15	Nil
16	Nil
17	Nil
18	Nil
19	Nil
20	Nil
21	Nil
22	**Tuberculous (TB) adenitis, syphilis**
23	Psoriasis, Hypertension
24	Nil
25	Nil
26	Psychiatric problems, ischaemic heart disease, **genital HSV, herpes zoster**
27	Nil
28	Nil
29	Nil
30	Nil
31	Nil
32	**Syphilis**
33	**Syphilis**
34	**Repeated urinary tract infections**
35	Nil
36	Nil
37	Nil
38	Nil
39	Depression
40	Nil

41	type 2 diabetes with retinopathy, haemophilia, **hepatitis C virus infection**, ulcerative colitis
42	Depression
43	Irritable bowel syndrome
44	Nil
45	Dermatitis
46	**Pulmonary TB**, renal failure unknown cause
47	Glucose-6-phosphatase dehydrogenase deficiency
48	Nil
49	Nil
50	Gall bladder polyps
51	**Latent TB**
52	Nil
53	Nil
54	**Latent TB,** schizoaffective disorder
55	**TB adenitis**
56	Nil
57	TV
58	Depression
59	Nil
60	Nil
61	**Genital warts**
62	Nil
63	Nil
64	**Oral and anogenital candidiasis**
Discord Controllers	
1	Haemophilia, **hepatitis C virus infection**
2	**Herpes zoster, oral HSV infection, genital warts**
3	Nil
4	Nil
5	**Syphilis**, **scabies**, **gonococcal infection, shingles**
6	Nil
7	Nil
8	Supra-ventricular tachycardia, peripheral neuropathy due to ART
9	Nil
10	Sickle cell trait
11	Nil
12	Sinusitis, **syphilis**
13	Nil
14	**Hepatitis C virus infection**, **syphilis**, Gilbert's syndrome, dyspepsia (biopsy helicobacter pylori positive)
15	Mitral valve stenosis, tricuspid valve regurgitation
16	**Syphilis, genital warts**
17	Nil
18	Nil

Those conditions which could be considered to be opportunistic (infections and neoplastic conditions) are shown in bold.

APPENDIX IV

Abstract of Paper Describing this Work, Accepted for Publication in Journal of Acquired Immune Deficiency Syndrome

Full article included at the back of the thesis.

Journal of Acquired Immune Deficiency Syndrome. 2012;61:407–416. PMID: 22902723
Disease Progression in Human Immunodeficiency Virus-1 Infected Viremic Controllers.

Groves KC, Bibby DF, Clark DA, Isaksen A, Deayton JR, Anderson J, Orkin C, Stagg AJ, McKnight A.

Abstract

BACKGROUND:
The mechanism of CD4 T-cell decline in Human Immunodeficiency Virus-1 (HIV-1) infection is unclear, but the association with plasma viral RNA-load suggests viral replication is involved. Indeed, viremic controller patients with low RNA-loads typically maintain high CD4 T-cell counts. Within a local cohort of 86 viremic controllers, we identify a subgroup (18 'discord controllers') with low CD4 T-cell counts which present clinical uncertainty. The underlying mechanism accounting for CD4 T-cell decline in the face of low or undetectable plasma (RNA) viral load remains unresolved. The objective of this study was to investigate the viral and host immune system dynamics in discord controllers by measuring cellular HIV-1 DNA-load, T-cell populations and T-cell activation markers.

METHODS:
We compared discord controllers (RNA-load <2000 copies/ml, <450 CD4 T-cells/mm) with typical controllers (RNA-load <2000 copies/ml, >450 CD4 T-cells/mm) and progressors (RNA-load >10,000 copies/ml, <450 CD4 T-cells/mm). We quantified CD4/CD8 naïve/ central-memory/ effector-memory subsets (CD45RA/RO ± CD62L), activation levels (CD38HLA-DR) and HIV-1 DNA-load.

RESULTS:
Discord controllers resembled progressors showing high DNA-load, depletion of naïve CD4 T-cells and higher activation in all CD4 T-cell subsets, compared with typical controllers. They were similar to typical controllers with lower CD8 T-cell activation compared with progressors.

CONCLUSIONS:
Our data are consistent with a relationship between CD4 T-cell activation and disease progression. HIV-1 DNA-load may be a better marker of viral replication and disease progression than RNA-load. Lower level CD8 T-cell activation correlates with low RNA-load, but not with disease progression or DNA-load.

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Disease Progression in HIV-1–Infected Viremic Controllers

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Background: The mechanism of $CD4^+$ T-cell decline in HIV-1 infection is unclear, but the association with plasma viral RNA load suggests viral replication is involved. Indeed, viremic controller patients with low viral RNA loads typically maintain high $CD4^+$ T-cell counts. Within a local cohort of 86 viremic controllers, we identify a subgroup (18 "discord controllers") with low $CD4^+$ T-cell counts that present clinical uncertainty. The underlying mechanism accounting for $CD4^+$ T-cell decline in the face of low or undetectable plasma (RNA) viral load remains unresolved. The objective of this study was to investigate the viral and host immune system dynamics in discord controllers by measuring cellular HIV-1 DNA load, T-cell populations, and T-cell activation markers.

Methods: We compared discord controllers (viral RNA load $<$2000 copies/mL, $<$450 $CD4^+$ T-cells/mm^3) with typical controllers (viral RNA load $<$2000 copies/mL, $>$450 $CD4^+$ T-cells/mm^3) and progressors (viral RNA load $>$10,000 copies/mL, $<$450 $CD4^+$ T-cells/mm^3). We quantified $CD4^+$/$CD8^+$ naive/central memory/effector memory subsets (CD45RA/RO ± CD62L), activation levels ($CD38^+HLA\text{-}DR^+$), and HIV-1 DNA load.

Results: Discord controllers resembled progressors showing high viral DNA load, depletion of naive $CD4^+$ T-cells, and higher activation in all $CD4^+$ T-cell subsets, compared with typical controllers. They were similar to typical controllers with lower $CD8^+$ T-cell activation compared with progressors.

Conclusions: Our data are consistent with a relationship between $CD4^+$ T-cell activation and disease progression. HIV-1 DNA load may be a better marker of viral replication and disease progression than viral RNA load. Lower level $CD8^+$ T-cell activation correlates with low viral RNA load but not with disease progression or viral DNA load.

Key Words: HIV-1, viremic controller, HIV-1 DNA, plasma RNA load, activation

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INTRODUCTION

After HIV-1 seroconversion, $CD4^+$ T-cells, the main target cell population, decline over the asymptomatic phase leading to progressive immunodeficiency. Although it is known that HIV-1 is cytopathic for $CD4^+$ T-cells, the mechanisms leading to $CD4^+$ T-cell depletion are not fully understood. It is suggested that the proportion of infected $CD4^+$ T-cells is too small to fully account for the extent of $CD4^+$ T-cell decline,[1] and an increased death rate is observed in nontarget cells, such as $CD8^+$ T-cells.[2] In keeping with this, Rodriguez et al[3] showed that in untreated individuals, plasma HIV-1 RNA load only minimally predicts $CD4^+$ T-cell decline, with current evidence suggesting that immune activation is the major predictor of disease progression.[4–11] Studies also suggest that naive T-cell compartments are preferentially depleted in progressive disease[10,12,13]; it is not known whether this is due to impaired production, sequestration to another site or phenotype, or destruction of naive T-cells. In addition, HIV-1 DNA load has been shown to predict disease progression.[14–16]

Some patients, viremic controllers, maintain low plasma viral RNA loads without antiviral medications. The vast majority of these patients are infected with replication-competent virus, and yet typically maintain high $CD4^+$ T-cell counts.[17] However, cases of progressive $CD4^+$ T-cell depletion despite continued control of viral RNA load have been described.[18–20] We term these patients "discord controllers" to reflect the discrepancy in viral RNA load and $CD4^+$ T-cell counts. This patient subset presents uncertainty in clinical management because parameters that are normally associated with progression (viral RNA load and $CD4^+$ T-cell count) are uncoupled. The mechanism leading to this discrepancy is unclear, although aberrant immune activation causing T-cell decline in the face of low plasma viral RNA load is one potential explanation.

We hypothesized that those changes typically associated with disease progression, depleted naive $CD4^+$ and $CD8^+$ T-cells, high level $CD4^+$ and $CD8^+$ T-cell activation, and high

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HIV-1 DNA loads, would be more marked in discord controllers as compared with typical controllers (low viral RNA load, high $CD4^+$ T-cells). Furthermore, we hypothesized that analysis of discord controllers with comparison to typical controllers would allow immunologic changes associated with disease progression and those due to high plasma viral RNA load to be distinguished; this is not possible with analysis of patients with the typical pattern of progression (high HIV-1 RNA load, low $CD4^+$ T-cells). Our aims based on these hypotheses were to analyze these factors typically associated with disease progression in discord controllers as compared with typical controllers and patients with a typical progression pattern.

METHODS

Study Groups

Patients were recruited, with informed written consent, from outpatient clinics at Barts and The London and Homerton University Hospital Trusts. All patients were HIV-1 seropositive and HIV-2 seronegative. Serology was not available for 1 patient, but sequence analysis as part of antiretroviral resistance testing demonstrated HIV-1 infection. Viremic controllers were defined as having plasma HIV-1 RNA loads below 2000 copies per milliliter [≥3 measurements over 12 months without antiretroviral therapy (ART)], definition in line with that used by the International HIV Controller Consortium. The controller cohort was divided into 2 groups based on the geometric mean of the last 3 $CD4^+$ T-cell counts: typical controllers >450 cells/μL (mm^3), discord controllers <450 cells/mm^3. The reason 450 cells/mm^3 was used as the cutoff was based on the fact many laboratories define a normal $CD4^+$ T-cell count to be above this value.[21] In addition, the "When to start consortium"[22] have found decreased mortality when ART is started in the range 351–450 cells/mm^3 instead of waiting until they are below 350 cells/mm^3, further supporting the suggestion that this range is abnormally low. The geometric mean of the 3 most recent % $CD4^+$ T-cells for each controller cohort was calculated to determine whether the lower absolute $CD4^+$ T-cell count in the discord controllers was reflected in a lower % $CD4^+$ T-cells. Epidemiological data were collected including age, sex, time since first positive test, ethnicity, country of birth, and bloodborne virus risk behavior.

Controls were (1) Viremic noncontrollers, for viral DNA load studies: ART-naive HIV-1–infected persons, infected >12 months with a viral RNA load >10,000 copies per milliliter; for T-cell studies controls comprised (2) HIV-1 progressors, definition as for viremic noncontrollers but with $CD4^+$ T-cell counts <450 cells/mm^3, and (3) uninfected subjects.

Plasma HIV-1 RNA loads and $CD4^+$ T-cell counts were stable for most controller patients. Two typical controllers, however, with low viral RNA load for 3.7 and 3.5 years subsequently showed a significant rise in viral RNA load while maintaining good $CD4^+$ T-cell counts (geometric means of 732 and 708 cells/mm^3, respectively). Another patient with low viral RNA loads and good $CD4^+$ T-cell counts for 2.3 years subsequently experienced a fall in $CD4^+$ T-cell count, but without an viral RNA load measurement, prompting initiation of ART. Thirteen patients (3 discord controllers and 10 typical controllers) were treated with ART during pregnancy but controlled plasma viral RNA load for at least 12 months both before and after ART. Controllers mentioned in this paragraph are included in the analysis of patient characteristics and clade analysis but were not used for viral DNA load and T-cell analysis. In addition, T-cell work was carried out only on those discord controllers with $CD4^+$ T-cell geometric mean of ≤410 cells/mm^3, and those typical controllers with a mean of ≥500 cells/mm^3, to avoid those patients too near the cutoff.

$CD4^+$ T-Cell Counts and Viral RNA Loads

Routine laboratory $CD4^+$ T-cell counts were performed using BD FACS Sample Prep Assistant with Trucount beads, acquiring samples on a BD FACSCanto II flow cytometer. FACSCanto clinical software was used for data analysis. Plasma viral RNA load was measured using Roche Ampliprep/COBAS Taqman HIV-1 Test v1.0 (Roche Molecular Systems, Inc, Pleasanton, CA; detection limit of 40 copies/mL). At least 1 sample from each patient was also tested using a different assay to confirm the low viral RNA load. Individual patient viral RNA loads and $CD4^+$ T-cell counts are reported as the geometric mean of last 3 measurements or last 3 before ART. If a patient had achieved viremic control (<2000 copies/mL, >12 months), but subsequently viral RNA load rose above 2000 copies per milliliter, the last date of viremic control was determined to be the last date an viral RNA load measurement would still contribute to a geometric mean of <2000 copies per milliliter.

Rate of change of $CD4^+$ T-cell count was calculated using the geometric mean of the 3 most recent values (x) and the mean of the oldest recorded values (y), and the time between them (z): $[(y - x)/z]$. Patients were only included in this calculation if the values spanned at least 3 years.

Clade Analysis

Some patients' clades were obtained during routine genotypic antiretroviral resistance testing (RNA sequence from protease and AA 1–335 of reverse transcriptase). This assay has demonstrated efficacy in picking up a wide variety of subtypes including recombinant subtypes.[23] For patients on whom genotypic antiretroviral resistance testing was unavailable, previously described *env*[24] and *gag*[25] polymerase chain reactions (PCRs) were used in parallel to accurately determine recombinant subtypes. Amplified products were sequenced using the second round forward and reverse primers from each PCR on an ABI 3100 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA). Sequences were analyzed using SeqScape V2.1.1 software and subtyped using the REGA online typing tool (Stanford University, 2006).

HIV-1 DNA Load Determination

DNA was extracted from cryopreserved peripheral blood mononuclear cells using Qiagen EZ1 DNA Blood Kit on the Qiagen BioRobot EZ1. A fixed concentration of phocine

herpes virus was added to each sample before extraction as an internal amplification control. A quantitative HIV-1 PCR, amplifying the long-terminal repeat (LTR) region, was designed based on previously described reverse transcriptase–polymerase chain reaction.[26,27] A final 25 μL PCR mixture contained 5 μL of peripheral blood mononuclear cells DNA, 2× PCR QuantiTect Multiplex RT-PCR No Rox Mix (Qiagen), 0.2 μM of each primer (F1 5′-AGCCTCAATAAAGCTTGCCTTGA-3′; R1 5′-GGCGCCACTGCTAGAGATTTT-3′), and 0.2 μM probe (AAGTAGTGTGTGCCCGTCTGT, fluorescent label). Thermocycling conditions (ABI 7500) were 95°C for 15 minutes, then 45 cycles of 95°C for 15 seconds, 60°C for 1 minute, and 72°C for 5 minutes. Mean values were calculated from duplicate PCRs. Quantitative PCR for β-globin (adapted from Lo et al[28]) was run in parallel. An external plasmid dilution series was run to construct a standard curve allowing viral DNA loads to be expressed as per cell equivalent.

T-cell Immunophenotyping Using Flow Cytometry

Seven-color flow cytometry was used to quantify $CD4^+$ and $CD8^+$ T-cell populations and their naive ($CD45RA^+CD62L^+$), central memory ($CD45RO^+CD62L^+$), and effector memory ($CD45RO^+CD62L^-$) subsets in EDTA-anticoagulated blood. Activation levels were determined by measuring the percentage of T-cells coexpressing CD38 and HLA-DR. The following monoclonal antibodies and fluorochromes were used: CD3-Pacific Blue, CD4-allophycocyanin (APC)-H7, CD45-RA-phycoerythrin(PE)-Cy7, CD45-RO-PE-Cy7, CD62L-PE, HLA-DR-peridinin chlorophyll protein (PerCP), CD38-PerCP-Cy5.5 (BD Biosciences, San Jose, CA).

Saturating amounts of monoclonal antibodies/isotype controls were incubated with 100 μL of whole blood (within 4 hours of collection) for 15 minutes at room temperature. Optilyse C (Immunotech, Marseilles, France) was added for 15 minutes at room temperature. Blood aliquots with single-color staining were processed with each sample for compensation. After 2 washes with phosphate-buffered saline containing 2% fetal calf serum, 0.02% NaN_3, and 1 mM EDTA, stained cells were resuspended and fixed in 300 μL phosphate-buffered saline 4% paraformaldehyde. A constant volume of Flow-count Fluorospheres (Beckman Coulter, Brea, CA) was added to enable absolute quantification of cells. The fixed stained cells were acquired within 12 hours of fixing on a BD LSR II cell analyzer (BD Biosciences) with a minimum of 50,000 (usually 100,000) events acquired per sample.

Winlist 6.0 software (Verity Software House, Topsham, ME) was used for analyses and color compensation (gating strategy shown in Fig. 1). $CD4^+$ T-cells were identified within a viable cell gate, set on the basis of light scatter, as cells that were $CD3^+CD4^+$. $CD8^+$ T-cells were identified as mononuclear cells that were $CD3^+CD4^-$. Two-dimensional dot plots, in which quadrant gates were set on the isotype controls, were used to define naive, central memory, and effector memory populations in both $CD4^+$ and $CD8^+$ T-cell populations. In turn, each of these populations were viewed on further 2-dimensional plots to determine activation level (percentage of cells coexpressing CD38 and HLA-DR). The number of flow-count spheres acquired allowed a precise determination of sample volume acquired, allowing expression of number of cells per volume of blood.

Statistical Analysis

Due to the rarity of these patients even in a large total HIV-1–infected cohort, power calculations were not undertaken to determine sample sizes; instead, all patients fulfilling the controller inclusion criteria and available to consent to the study were included. Therefore, it is necessary to interpret non-statistically different results with caution.

Analysis was performed using Prism (version 4.0; Graphpad Software, San Diego, CA) and results considered significant if $P < 0.05$. Viral RNA and DNA loads were $\log_{10}$ transformed before statistical analysis. To determine differences in HIV-1 RNA/DNA loads, age, time since first positive test and T-cell flow data, a 2-tailed Mann–Whitney U test was applied. Fisher's exact test (2-tailed) was used when comparing sex distribution, ethnicity, country of birth, risk behavior, and clade in each cohort. A correction for multiple comparisons was employed using the false discovery rate calculation.

RESULTS

Controller Phenotype

A cohort of 82 HIV-1 viremic controllers was established: 64 typical controllers and 18 discord controllers (Table 1). Approximately 3000 patients attend the clinics, thus viremic controllers represent 2.7% (discord controllers 0.6%, typical controllers 2.1%) of all infected patients. Comparing the 2 controller cohorts, plasma viral RNA loads were indistinguishable ($P = 0.71$). Nine typical controllers and 1 discord controller were elite controllers (viral RNA load below 50 copies/mL, occasional nonconsecutive blips). There was no difference in median age ($P = 0.44$), sex distribution ($P = 0.79$), ethnicity, region of birth, or risk behavior. No significant difference was found comparing time since first positive test (best available surrogate for time since seroconversion) between the 2 controller cohorts ($P = 0.25$), but a larger cohort may reveal a difference. The $\%CD4^+$ T-cells in the discord controllers [median 22.3, interquartile ratio (IQR) 17.6–26.0] was significantly lower than that seen in the typical controllers (median 33.7, IQR 23.8–40.0, $P < 0.0001$). The rate of change of $CD4^+$ T-cell count was not significantly different comparing the 2 cohorts (discord controllers, median −2.9, IQR −26.4 to −1.0; typical controllers, median 2.1, IQR −16.5 to −34.6, $P = 0.2174$); however, again, a larger study could reveal a difference here.

Clade Distribution in Controller Cohorts

The clade distribution in the East London HIV-1–infected population is diverse,[23] with around 64% of isolates being non-clade B (D. A. Clark, PhD, and D.F. Bibby, PhD, unpublished data). We investigated whether there was a skew regarding infecting viral clade in discord controllers. It was not possible

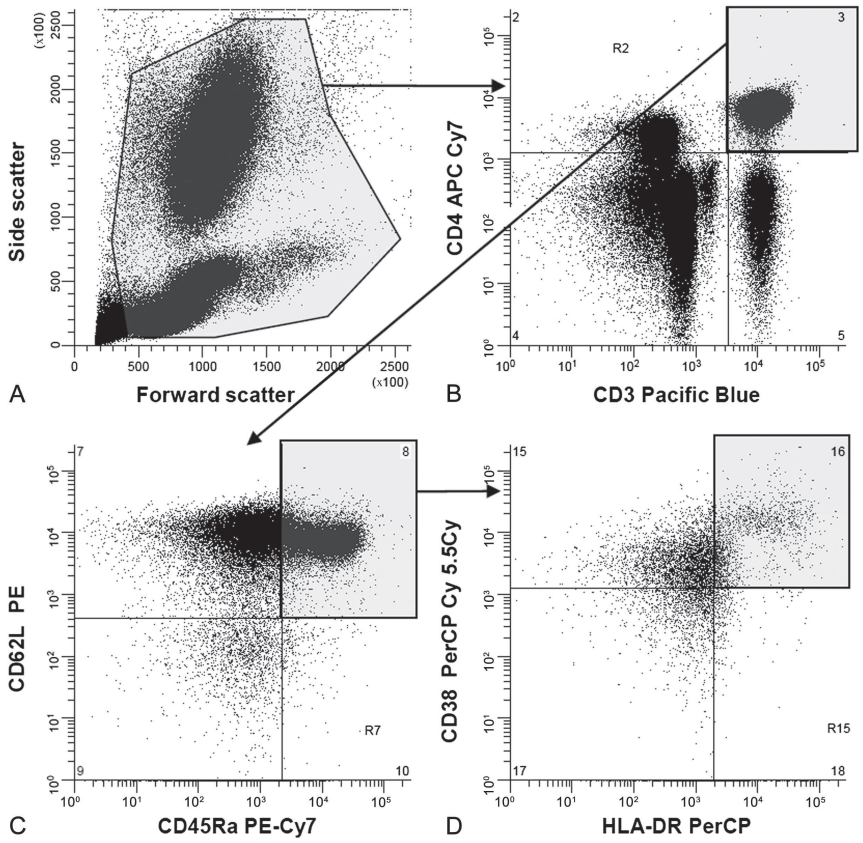

FIGURE 1. Gating strategy for flow cytometric analysis of T-cell subsets. A, Forward and side scatter to define viable cell gate. B, Double staining of viable cells with anti-CD3 and anti-CD4. $CD4^+$ T-cells are in the top right quadrant; $CD4^-$ T-cells (predominately $CD8^+$ T-cells) are in the bottom right quadrant. C, Double staining of $CD4^+$ T-cells with anti-CD45RA and anti-CD62L, naive $CD62L^+CD45RA^+$ T-cells, defined with reference to staining with isotype-matched control antibodies, are in the top right quadrant. D, Double staining of naive $CD4^+$ T-cells with anti-CD38 and anti-HLA-DR, activated $CD38^+HLA\text{-}DR^+$ T-cells, defined with reference to staining with isotype-matched control antibodies, are in the upper right quadrant.

to obtain a product for 2 samples tested (plasma viral RNA load <50 and 111 copies/mL). As shown in Table 1, there was a non-significant trend towards an increased frequency of clade C in the discord controllers (40.0%) compared with typical controllers (17.1%) and also compared with the total tested HIV-1 population (25.1%) (D. A. Clark, PhD, and D.F. Bibby, PhD, unpublished data over 5 years at Department of Virology, Barts and the London NHS Trust).

Depleted Naive $CD4^+$ T-Cell Compartment in Discord Controllers

HIV-1 infection, as it progresses, leads to decline of $CD4^+$ naive T-cells.[10,12] We measured the number and percentage of these naive cells ($CD45RA^+CD62L^+$), central memory T-cells ($CD45RO^+CD62L^+$), and effector memory T-cells ($CD45RO^+CD62L^-$) in blood from participants in each cohort.

As expected, lower $CD4^+$ T-cell numbers were seen in each subset in progressors and discord controllers compared with typical controllers and uninfected patients (Figs. 2A–C). A more marked depletion of the naive subset was seen in both discord controllers and progressors compared with the other patient groups (percentage of total $CD4^+$ T-cells, Fig. 2D). The higher percentage of effector memory cells in progressors and discord controllers compared with typical controllers (Figs. 2C, F) represents a reciprocal change due to marked loss of naive cells. The percentage of central memory T-cells was similar in all 3 patient groups (Fig. 2E).

Increased $CD4^+$ T-Cell Activation in Discord Controllers

High-level $CD4^+$ T-cell activation is associated with disease progression.[5,8] We, therefore, measured T-cell activation level (percentage of cells coexpressing $CD38^+$ and $HLA\text{-}DR^+$) in blood. Progressors and discord controllers had comparably increased levels of activation in naive, central memory, and effector memory $CD4^+$ T-cell subsets (Figs. 2G–I) compared with typical controllers and uninfected subjects. Typical controllers demonstrated a trend toward increased activated effector memory $CD4^+$ T-cells as previously reported.[29]

Naive $CD8^+$ T-Cell Levels Are Not Depleted Compared With Typical Controllers

So far, our results suggest that the $CD4^+$ T-cell compartment is similar in discord controllers and progressors. We next characterized the $CD8^+$ T-cell population.

TABLE 1. Patient Characteristics and Clade of Infecting Virus in Controller Cohorts

	Typical Controllers ($CD4^+$ Count >450 cells/mm^3), n = 64	Discord Controllers ($CD4^+$ Count <450 cells/mm^3), n = 18*
Age, yrs	37.5 (32.0–43.0)	38.5 (32.5–49.0)
Female sex, n (%)	35 (54.7)	11 (61.1)
$CD4^+$ T-cell count, cells/mm^3†	699.3 (550.4–843.1)	347.2 (298.0–406.6)
Plasma viral RNA load, $\log_{10}$ copies/mL†	408.5 (160.7–1000.0)	428.6 (100.3–1043.0)
Place of birth, n (%):		
Europe	25 (39.1)	7 (38.9)
Africa (Sub-Sahara)	28 (43.7)	10 (55.6)
Caribbean	6 (9.4)	0 (0)
South Asia	3 (4.7)	0 (0)
Other	2 (3.1) (Australia, Ecuador)	1 (5.5) (New Zealand)
Ethnicity, n (%)		
White British	12 (18.8)	3 (16.7)
White other	8 (12.5)	2 (11.1)
Black African (Sub Saharan)	28 (43.7)	11 (61.1)
Black Caribbean	11 (17.2)	2 (11.1)
South Asian	3 (4.7)	0 (0)
Other	2 (3.1) (Mixed white and black African; South American)	0 (0)
Time since first positive test, yrs	4.5 (2.5–6.5)	4.6 (3.3–9.3)
Risk behavior		
Heterosexual	42 (65.6)	11 (61.1)
Specified abroad	26 (40.6)	5 (27.8)
Homosexual	20 (31.3)	6 (33.3)
Blood Products	1 (1.6)	1 (5.6)
IVDU	1 (1.6)	0 (0)
Clade, n‡ (%)	n = 41	n = 15
B	14 (34.1)	6 (40.0)
CRF02_AG	8 (19.5)	3 (20.0)§
C	7 (17.1)	6 (40.0)
A	4 (9.7)	0 (0)
F	2 (4.9)	0 (0)
CRF14_BG	2 (4.9)	0 (0)
Other	D; A/C; A/D; B/D	0 (0)

Data presented in this table, when not stated to be n (%) are median (IQR).

*Three patients in this cohort started ART due to their low $CD4^+$ T-cell count (viral RNA load/$CD4^+$ values before starting ART were used).

†Median (IQR) of geometric mean calculated for each subject using 3 most recent $CD4^+$ T-cell count and plasma viral RNA load values.

‡Data obtained using genotypic antiretroviral resistance testing for 28 typical controllers and 10 discord controllers, and using nested PCR for 13 typical controllers and 5 discord controllers; a product was not amplified for 2 samples tested using the nested PCRs (plasma viral RNA load <50 and 111 copies/mL).

§For 1 sample, sequence analysis of nested PCR products indicated either CRF02_AG or CRF09_CPX. Statistical analysis employed a Mann–Whitney *U* test for plasma viral RNA load, age, time since first positive test, and a Fisher's exact test for sex distribution, ethnicity, country of birth, and clade in each cohort. IVDU indicates intravenous drug use.

Controller groups had similar numbers and percentages of $CD8^+$ naive T-cells compared with uninfected controls, whereas the progressors had a marked depletion of this cell type (Figs. 3A, D). Progressors had an expanded number of effector memory $CD8^+$ T-cells compared with all other groups (Fig. 3F).

$CD8^+$ T-Cell Activation Is Not Higher in Discord Controllers Compared With Typical Controllers

$CD8^+$ T-cell activation ($CD38^+$ $HLA\text{-}DR^+$) was increased in all T-cell subsets of all HIV-positive patients, even in typical controllers, compared with uninfected controls as has been demonstrated by Lopez et al.[30] This is in keeping with the proposition that $CD8^+$ T-cell subset alterations are a highly sensitive marker of HIV-1 infection. This increased activation was more marked in progressors in all subsets, especially in the memory compartments (Figs. 3G–I); in keeping with a previous report showing increased memory $CD8^+$ T-cell activation in noncontrollers compared with controllers.[31] In comparison, activation was equivalently low in both controller cohorts (Figs. 3G–I). Thus, despite the fact that discord controllers have low $CD4^+$ T-cell counts (like progressors), their $CD8^+$ T-cell activation pattern more closely resembles that of typical controllers.

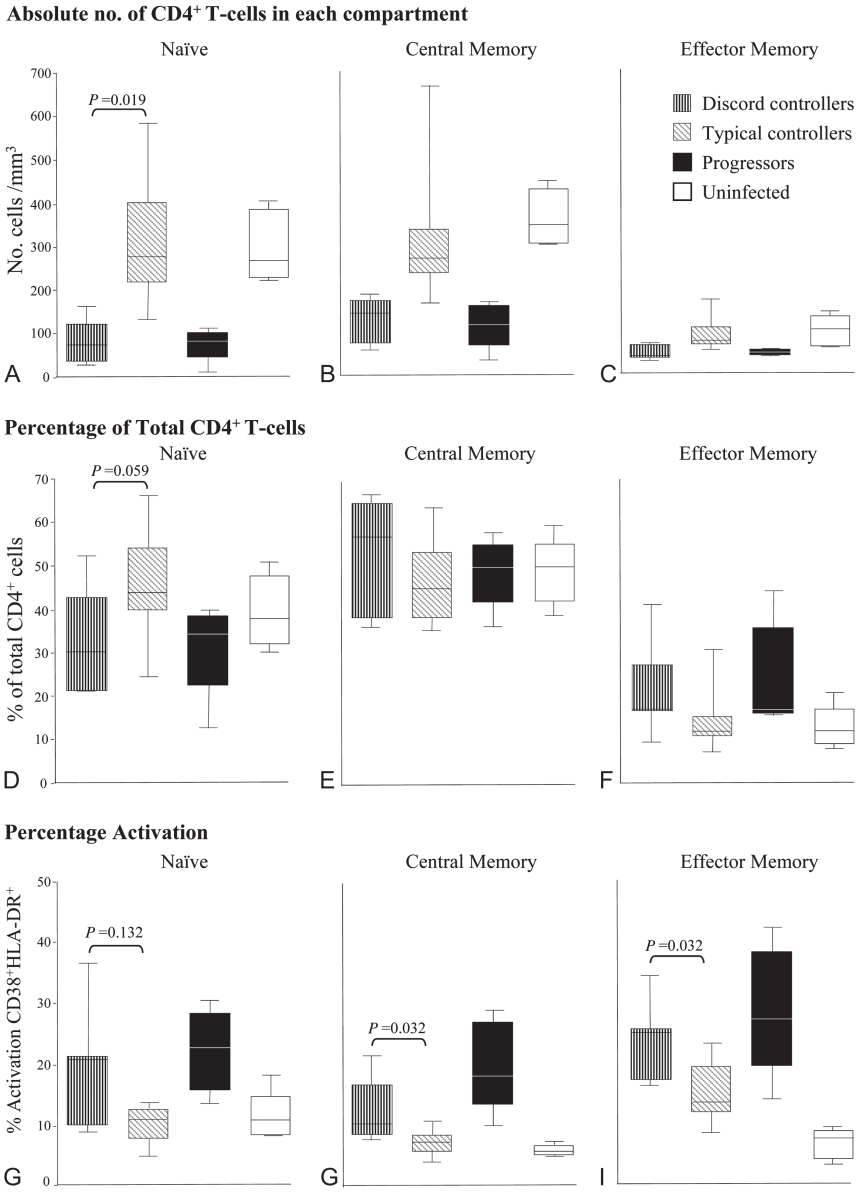

FIGURE 2. Representation of CD4+ T-cell subsets by patient group. Patients analyzed included 7 patients with discord controller phenotype [median (IQR) for viral RNA load 675 (128–1320) copies/mL, CD4+ T-cells/mm3 337 (267–400)]; 12 patients showing the typical controller phenotype [viral RNA load 268 (116–926) copies/mL, CD4+ T-cells/mm3 815 (691–971)], 5 progressors [viral RNA load 60,683 (28,603–155,518) copies/mL, CD4+ T-cells/mm3 309 (296–339)], and 5 uninfected subjects. Progressors: age 35 (30–48) years, female 40%, CD4+ T-cell count 309 (287–346) cells/mm3, HIV-1 RNA load 60,682 (23,947–232,854) copies per milliliter, all reported as median (range). Absolute numbers of cells in CD4+ T-cell subsets are shown in (A) naive (CD45RA+CD62L+), (B) central memory (CD45RO+CD62L+), and (C) effector memory (CD45RO+CD62L−). Percentage of cells in CD4+ T-cell subsets compared with total CD4+ T-cell pool are shown for (D) naive, (E) central memory, and (F) effector memory. Percentage activation of cells in CD4+ T-cell subsets (as percentage of total cells in that subset) by patient group is shown in (G–I). Each central bar represents the median value, each box represents the IQR, and the whiskers represent the minimum and maximum values. Statistical analysis employed a Mann–Whitney *U* test.

Higher HIV-1 DNA Load in the Discord Controller Cohort Compared With Typical Controllers

Viremic controllers typically have low HIV-1 DNA loads in addition to low viral RNA loads.[32] Because discord controllers have low CD4+ T-cell counts, we hypothesized that high levels of viral replication were nevertheless ongoing and that this may be reflected in higher viral DNA loads. Plasma viral RNA load and cellular viral DNA load were determined for controllers and a group of noncontrollers (Fig. 4). We found discord controllers to have significantly higher viral DNA loads (median 601, range 13–1529 copies per 10^6 CD4+ T-cells) than typical controllers (median 87, range 0–755 copies per 10^6 CD4+ T-cells) and that viral DNA loads were as high as those seen in noncontrollers (high viral RNA loads) (median 852, range 27–2188 copies per 10^6 CD4+ T-cells), suggesting significant ongoing viral replication.

ART in Discord Controllers Leads to Modest Recovery of CD4+ T-Cell Counts

Typically, after initiation of ART in a patient, plasma viral RNA load becomes undetectable and CD4+ T-cell count

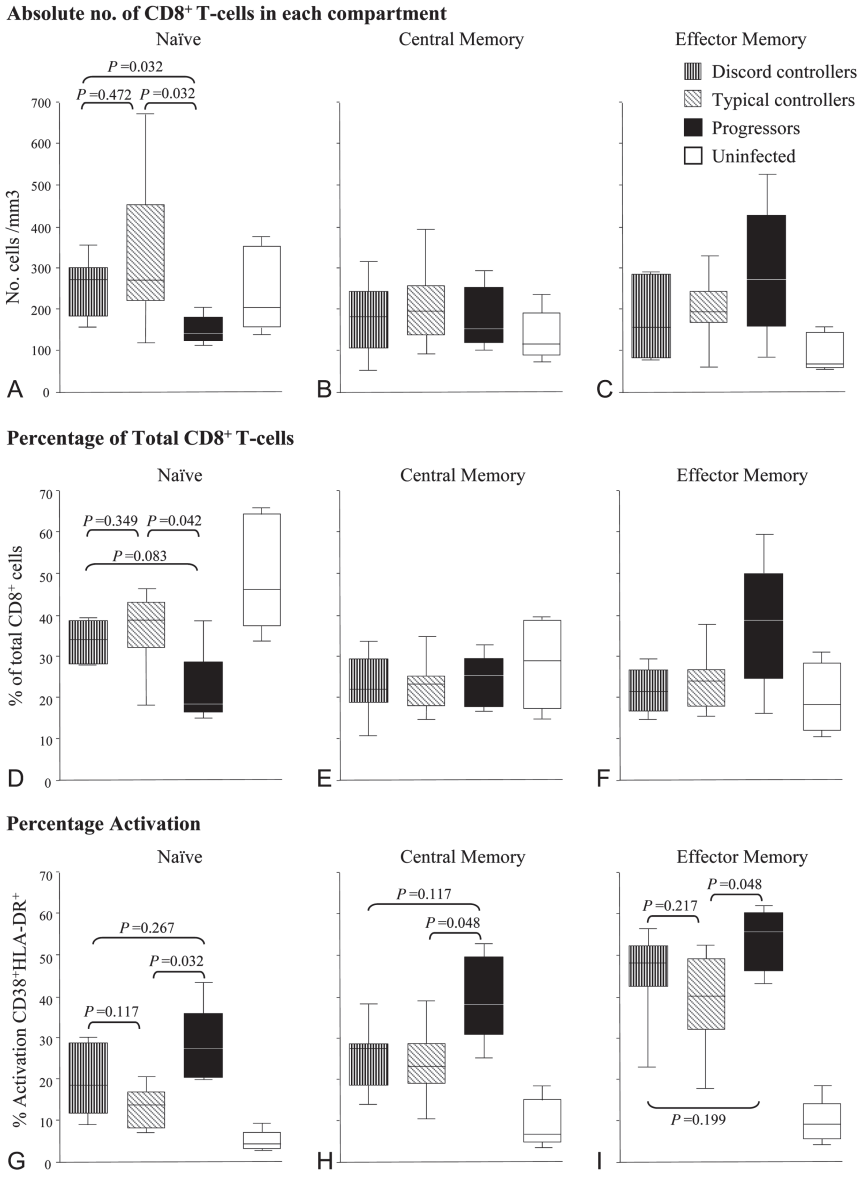

FIGURE 3. Representation of CD8+ T-cell subsets by patient group. Patients analyzed as described in Figure 2: discord controllers, typical controllers, progressors, and uninfected subjects. Absolute numbers of cells in CD8+ T-cell subsets are shown in (A) naive (CD45RA+CD62L+), (B) central memory (CD45RO+CD62L+), and (C) effector memory (CD45RO+CD62L−). Percentage of cells in CD8+ T-cell subsets compared with total CD8+ T-cell pool are shown for (D) naive, (E) central memory, and (F) effector memory. Percentage activation of cells in CD8+ T-cell subsets (as percentage of total cells in that subset) by patient group is shown in (G–I). Each central bar represents the median value, each box represents the IQR, and the whiskers represent the minimum and maximum values. Statistical analysis employed a Mann–Whitney *U* test.

increases.[33] During this study, 5 patients in the discord controller group started ART. This resulted in an increase from 308 to 353 CD4+ cells/mm3 over 12 months of ART, 269 to 319 CD4+ cells/mm3 over 19 months, 249 to 414 CD4+ cells/mm3 over 19 months, 269 to 460 CD4+ cells/mm3 over 23 months, and 284 to 441 CD4+ cells/mm3 over 22 months of ART. An early rise was seen for only 1 patient where CD4+ T-cell count rose from 249 to 411 cells/mm3 after just 1 month of ART; CD4+ T-cell recovery occurred over many months for the other patients, and indeed counts continued to increase past 12 months of ART for 2 of the patients.

It was observed that although absolute CD4+ T-cell counts rose early for only 1 patient, %CD4+ T-cells did rise early for 3 patients due to the fact that absolute CD8+ T-cell count dropped markedly after 1 month of ART (760–536, 1429–1005, and 892–421 cells/mm3). Other studies have described a decrease in either memory CD8+ T-cells,[34] activated CD8+ T-cells,[35] or HIV-1-specific CD8+ T-cells[36] within the first few weeks of ART that may account for the decrease we see in total CD8+ T-cells.

In summary, discord controllers share many characteristics with progressors, namely high HIV-1 DNA loads, a depleted naive CD4+ T-cell population with increased CD4+ T-cell activation levels; in contrast, the CD8+ T-cell compartment in discord controllers is more similar to that seen in typical controllers.

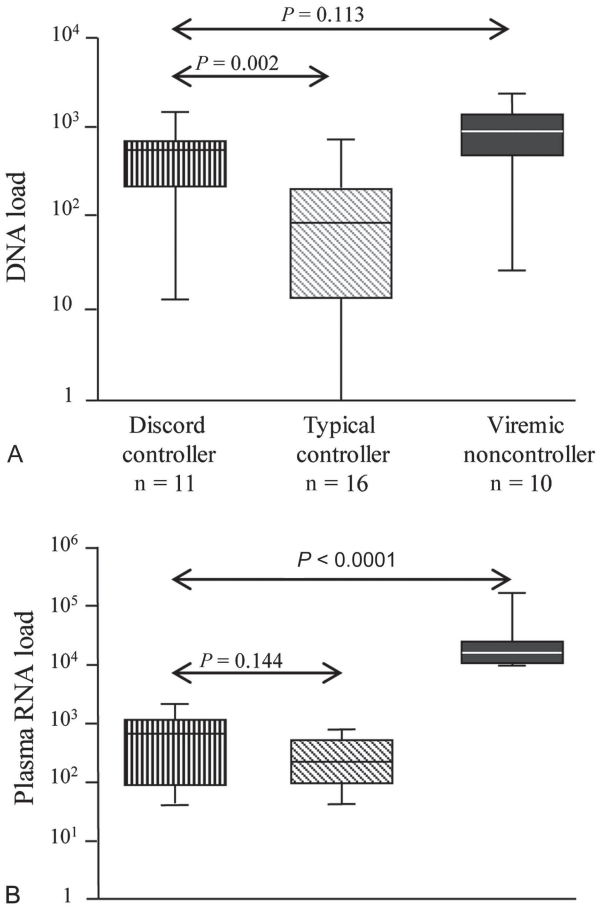

FIGURE 4. HIV-1 DNA load compared with HIV-1 RNA load in patient groups. A, viral DNA load (DNA copies per 10^6 CD4+ T-cells; $\log_{10}$ scale) as compared with B, Plasma viral RNA load (copies/mL; $\log_{10}$ scale) by patient group. Each central bar represents the median value, each box represents the IQR, and the whiskers represent the minimum and maximum values. Viremic noncontrollers: age 42 (28–66) years, female 10%, CD4+ T-cell count 399 (258–716) cells/mm^3, HIV-1 RNA load 15,809 (10,041–169,049) copies per milliliter, all reported as median (range). Median (range) of CD4+ T-cell count values (cells/mm^3) within 12 months before the sample tested for plasma HIV-1 DNA load were 339 (251–424) for discord controllers; 775 (499–1326) for typical controllers; 399 (258–716) for viremic noncontrollers. Statistical analysis employed a Mann–Whitney *U* test.

DISCUSSION

In HIV-1 infection, low viral RNA loads usually predict good CD4+ T-cell counts.[37] We describe a cohort of HIV-1–infected patients, who, despite maintaining low viral RNA loads (similar to typical controllers), have low CD4+ T-cell counts (similar to typical progressors) indicating disease progression. We show that these patients, termed discord controllers, in addition to having low CD4+ T-cell counts have high viral DNA loads, preferentially depleted naive CD4+ T-cells and increased CD4+ T-cell activation, similar to typical progressors. This finding supports Hunt et al[18] who show that lower CD4+ T-cell counts correlate with higher CD4+ T-cell activation even among controllers. Of note, the increased CD4+ T-cell activation was similar in discord controllers and progressors, despite progressors having much higher plasma viral RNA loads of the latter groups, suggesting that factors independent of viral RNA load are driving CD4+ T-cell activation.

With regard to the CD8+ T-cell compartment, discord controllers were more similar to typical controllers. Both controller cohorts have higher numbers of naive CD8+ T-cells and lower levels of CD8+ T-cell activation compared with progressors. These observations suggest that a more preserved CD8+ T-cell compartment is associated with control of plasma viremia but not necessarily with lack of disease progression as is shown in discord controllers. Some studies suggest that CD8+ T-cell activation is a predictor of disease progression, independent of CD4+ T-cell count and HIV-1 RNA.[4,6,38] However, we do not find this to be the case in our controller population. Indeed, our observations are more in keeping with alternate studies, which show that higher plasma viral RNA load positively correlates with increased CD8+ T-cell activation,[5,30] and that this correlation holds regardless of CD4+ T-cell counts.[30] Functional analysis of the CD8+ T-cells present in these patient cohorts may highlight differences in cellular response to HIV-1, which confer protection against disease progression.

There was no association between the discord phenotype and age or time since first positive test (best available surrogate for time since seroconversion). Although time since diagnosis is not a perfect surrogate for time since seroconversion, this data suggests that the discord controller phenotype is not associated with a longer duration of infection compared with typical controllers. However, this study was not powered or designed to determine this fully. The fact that there is a trend toward lower %CD4+ T-cells in the discord controller cohort argues against suggestions that lower absolute CD4+ T-cell counts in the discord controller group are merely a feature of lower total lymphocyte counts associated with ethnic variation. The finding that the rate of change of CD4+ T-cell count was indistinguishable comparing the 2 controller cohorts might also support this premise. There was a trend toward an over-representation of clade C in the discord cohort; however, larger studies are required to confirm this association and determine whether this clade predisposes to the discord controller phenotype.

Initiation of ART in 5 patients achieved a modest gain in CD4+ T-cell numbers similar to those described recently by Okulicz et al[39] further supporting the notion that low CD4+ T-cell counts in the discord controller population are not merely due to a lower "normal" CD4+ T-cell count, for example, due to ethnic variation. An increase over the first 10 weeks of ART has been suggested to be due to "redistribution" of CD4+ T-cells trapped in lymphoid.[40] The fact that this type of early rise occurred for only 1 of the 5 patients suggests that redistribution was not the predominant mechanism leading to CD4+ T-cell recovery in these patients.

Unintegrated HIV-1 DNA is less stable than integrated "proviral" DNA, and as such has been proposed, together

with total HIV-1 DNA, to be a marker for ongoing replication in vivo.[41] This notion is further supported by observations that DNA reservoirs are maintained by viral replication that may be suppressed by ART[42,43] and that total HIV-1 DNA correlates inversely with $CD4^+$ T-cell counts and predicts disease progression.[15,16] Compared with typical controllers, we found significantly higher viral DNA loads in the discord controllers, which was equivalent to that seen in progressors suggesting high levels of ongoing replication in vivo. It is unclear why this high cellular viral DNA load does not result in a higher-level plasma viral RNA load in the discord controllers. Given the crucial role of the $CD8^+$ compartment in control of plasma viral RNA load,[44–46] the low plasma viral RNA load in the discord controllers may be contingent on a $CD8^+$ T-cell compartment that is maintained in a subset distribution and activation pattern similar to typical controllers. Another possibility is that viral replication is strictly compartmentalized; the gastrointestinal tract (lamina propria and organized lymphoid tissue) is a potential candidate because it has been shown to be an important site of HIV-1 replication and of $CD4^+$ T-cell sequestration and depletion.[47] Alternatively, discord controllers may be infected with a virus that predominantly spreads directly from cell to cell. Another scenario is that virus may be released efficiently into the blood but cleared by humoral immune mechanisms. These possible scenarios that may not be mutually exclusive are currently under investigation.

With respect to the mechanism of $CD4^+$ T-cell loss in discord controllers, our observations favor a model where $CD4^+$ T-cell depletion is driven, at least partly, by $CD4^+$ T-cell activation. This activation seems to be associated with high-level viral replication as suggested by high total HIV-1 DNA loads. This is in keeping with a large body of evidence, which suggests that immune activation is associated with more rapid clinical progression and $CD4^+$ T-cell decline.[4–11] This may result from clonal exhaustion and drainage of memory T-cell pools. Concomitantly, naive $CD4^+$ T-cell depletion may result from this high immune activation driving cells into another phenotype but may also be due to a deficiency of $CD4^+$ T-cell regeneration or sequestration to another site. Sequential studies in individual patients are required to further elucidate this, given the dynamic nature of HIV-1 infection.

In summary, we have identified a subset of HIV-1–infected patients, discord controllers, who despite maintaining low plasma viral RNA loads experience disease progression. With the exception of $CD8^+$ T-cell subset distribution and activation, we show that discord controllers are similar to progressors, based on the $CD4^+$ T-cell compartment and HIV-1 DNA load, further supporting data that plasma viral RNA load alone does not lead to $CD4^+$ T-cell decline. Rather, we have demonstrated increased $CD4^+$ T-cell activation in discord controllers, which may relate to the higher viral DNA load in this group. This higher viral DNA load suggests comparatively higher levels of virus turnover in the discord controllers, despite controlled plasma viral RNA loads, possibly explaining disease progression. Moreover, the fact that spontaneous control of viral replication does not necessarily confer protection against disease progression highlights the need to intensify the search for new therapies aimed at normalizing perturbations of the T-cell compartment.

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