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THE ROLE OF INTEGRIN $\alpha_4\beta_7$ BINDING IN HIV-1 SUBTYPE C PATHOGENESIS IN PHENOTYPICALLY VARIANT $CD4^+$ T CELL SUBSETS

Simone Irene Richardson

A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree

of

Master of Science in Medicine

Johannesburg, 17 February 2014

Declaration

I, Simone Irene Richardson declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.



Dedication

This work is dedicated to my parents Caryll and Derrick Richardson who have poured their heart and soul into my well-being and education, never faltering in their faith in me.

Acknowledgements

Firstly it has been a privilege to be part of the HIV virology lab. Being surrounded by incredibly talented and hardworking individuals has motivated me to expand my horizons.

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Simone Richardson, Nonhlanhla Mkhize, Elin Gray, Lynn Morris. Expression of integrin $\alpha_4\beta_7$ on Treg and Th17 subsets indicates a novel route of HIV-1 dissemination. Oral presentation on 2-5 September 2012 at EMBO Global Exchange Lecture Course on Innate Immunity – evolution and advances in clinical medicine, Johannesburg, South Africa.

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Abstract

The integrin α4β7, which mediates the trafficking of T lymphocytes to the gut associated lymphoid tissue (GALT), a site of rapid HIV replication, has been described as an attachment factor for the HIV envelope protein gp120. While differences in binding affinity of early transmitting and chronic gp120s for α4β7 have previously been noted by others, this has not translated to differences in replication. We aimed to investigate what role this binding interaction has in HIV pathogenesis over time and determine the factors that influence α4β7 reactivity. Understanding the subsets on which α4β7 is expressed may indicate the phenotype of the first host cells infected by HIV. The level of expression of α4β7 on Th17 and Treg $CD4^+$ T cells was of interest as both subsets are important in HIV immunity in the GALT and represented in both the GALT and genital mucosa.

All-trans retinoic acid-activated $CD4^+$ T cells isolated from whole blood of healthy donors were incubated with or without HP2/1 (anti-α4 monoclonal antibody) or Act-1 (anti-α4β7 monoclonal antibody) prior to adding virus. Sixty infectious envelope clones were prepared using matched envelope genes from 11 individuals in the CAPRISA 002 Acute Infection cohort representing the T/F virus and variants from 1-39 months post infection (p.i.). Replication was monitored by p24 ELISA over 10 days. ATRA-stimulated $CD4^+$ T cells were subjected to intracellular and surface staining for flow cytometric analysis using a FACSAria to distinguish Th17 and Treg $CD4^+$ T cells and to determine the expression of CD45RA, CCR5, p24, α4β7.

The dependence on α4β7 for HIV subtype C replication changes over time and varies across individuals. In three individuals, dependence on α4β7 was higher using T/F viruses and decreased sharply during acute infection (1-3 months post-infection). α4β7 dependence showed an increasing trend in chronic infection over time which was slight in the first year. Factors that influence α4β7 reactivity include glycan distance from α4β7-binding motif, glycan density and length of the V1/V2 region of gp120. Several glycans positioned in conserved regions of gp120 including N234, N332 and N334 were present more or less frequently in viruses with high α4β7 reactivity. There was an

association between high dependence on α4β7 for replication at transmission and those T/F viruses that have a S/PDI/V α4β7 binding motif as well T/F viruses found in individuals diagnosed with bacterial vaginosis during acute infection. Several cytokines in the cervicovaginal lavage (CVL) of these individuals during early infection (IL-8, IL-7 and IL-1α) positively correlated with α4β7 dependence for replication. Treg $CD4^+$ T cells expressed slightly higher levels of α4β7 as compared to Th17 $CD4^+$ cells. In addition, Th17 cells in this and other studies were shown to rapidly deplete *in vitro* following HIV infection while Treg $CD4^+$ T cell were shown to expand. Despite this, Treg $CD4^+$ T cells were significantly more permissive to infection as compared to Th17 $CD4^+$ T cells.

Collectively, these data suggest that there is a role for α4β7 in HIV pathogenesis and that the interaction is selected for during transmission by a number of bottlenecks, one of which is the presence of bacterial vaginosis and elevated expression of IL-7, IL-8 and IL-1α. Due to high levels of α4β7 expression and the ability to bind gp120, presence in both the genital tract and the GALT, regulation by ATRA similar to α4β7 upregulation, expansion following HIV infection and elevated permissiveness to acute infection, Treg $CD4^+$ T cells may be a robust vector from the genital mucosa to the GALT shortly after transmission. This population of T cells may be more suited for this function than Th17 $CD4^+$ T cells which are more susceptible to depletion either by HIV or bystander effects. As a result of the α4β7 binding motif being in a highly immunogenic region of gp120 and antibodies directed against this region associated with protection in the only effective vaccine trial to date, further understanding of the interaction between the virus and the integrin provides an opportunity for the development of future vaccine and therapeutic strategies.

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Amino acid abbreviations

Amino Acid	**Three-Letter code**	**One-Letter code**
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

List of abbreviations

AIDS	Acquired Immune Deficiency Syndrome
APC	Allophycocyanin
ATRA	*All-trans* retinoic acid
BV	Bacterial vaginosis
BSA	Bovine serum albumin
C1, C2, C3, C4, C5	Conserved regions of gp120
C3-V4	The entire C3 and adjoining V4 region
CAPRISA	Centre for the AIDS Program of Research in South Africa
CAP	CAPRISA
CCR5	C-C motif chemokine receptor type 5
CD	Cluster of differentiation
CVL	Cervicovaginal lavage
CXCR4	C-X-C motif chemokine receptor type 4
DMEM	Dulbecco′s Modified Eagle′s Medium
DNA	Deoxyribonucleic acid
DMSO	Dimethylsulphoxide
ELISA	Enzyme-linked immunosorbent assay
Env	Envelope glycoprotein
FACS	Fluorescence activated cell sorting
FBS	Foetal bovine serum
FITC	Fluorescein isothyocyanate
FMO	Fluorescence minus one
FSC	Forward scatter
FSC-H	Forward scatter height
GALT	Gut associated lymphoid tissue
gp120	HIV envelope glycoprotein (120 kilodalton)
gp41	HIV envelope glycoprotein (41 kilodalton)
GVHD	Graph versus host disease
HEK 293T	Human embryonic kidney 293T cell line
HIV-1	Human Immunodeficiency Virus Type-1
IBD	Inflammatory bowel disease
ICAM-1	Inter-cellular adhesion molecule (CD54)
IEC	Infectious envelope clone
IL	Interleukin
IMC	Infectious molecular clone

LFA-1	Lymphocyte function associated integrin-1
mAb	Monoclonal antibody
MAdCAM-1	Mucosal addressin cell adhesion molecule- 1
MPER	Membrane proximal external region
P24	Gag protein 24KDa
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PerCP-Cy5.5	Peridininchlorophyll protein-Cy5.5
p.i	Post infection
PMA	Phorbol 12-myristate 13-acetate
PNGs	Potential N-linked glycosylation sites
Qdot	Quantum dot
RAR	Retinoic acid receptor
RLU	Relative light units
RPMI	Roswell Park Memorial Institute
RXR	Retinoid receptor X
SANBS	South Africa National Blood Service
SIV	Simian Immunodeficiency Virus
SGA	Single genome amplification
SSC	Side scatter
STATSSA	Statistics South Africa
STI	Sexually transmitted infections
TCID50	50% tissue culture infectious dose
T/F virus	Transmitted /founder virus
Th17	T helper-17 cells
Treg	T regulatory lymphocytes
V1, V2, V3, V4, V5	Variable loop regions of gp120
V1/V2	The entire V1 and adjoining V2 region
VCAM-1	Vascular cell adhesion molecule-1
UNAIDS	United Nations Programme on HIV/AIDS

CHAPTER 1

INTRODUCTION

1.1 The shape of the HIV/AIDS epidemic in South Africa and efforts to fight it

HIV/AIDS is the defining pandemic of the 21st century. An estimated 34 million people were infected with HIV at the turn of 2012 globally and Sub-Saharan Africa bears approximately 69% of the burden (UNAIDS, 2012). Although a declining trend in new HIV infections both globally and in Southern Africa was reported by UNAIDS in 2012, an astounding 71% of new infections are accounted for by this population, despite the implementation of prevention and awareness strategies. In South Africa, it is estimated that 5.38 million people are living with HIV, and with new infections estimated to total 316,900 people in 2011 (STATSSA, 2011), innovative strategies for the development of prevention methods are essential.

Current HIV behavioural and awareness teachings on abstinence, faithfulness and frequent condom use have had a limited impact on HIV incidence in South Africa. Several other prevention strategies have yielded measurable success with the most notable including a microbicide efficacy trial using a 1% formulation of the antiretroviral (ARV) drug, tenofovir (Abdool Karim et al., 2010), medical male circumcision (Auvert et al., 2005; Bailey et al., 2007; Gray et al., 2007b) and treatment of sexually transmitted diseases (Grosskurth et al., 1995). These successes yielded efficacies of 39%, 57% and 42% respectively in clinical trials with only circumcision and treatment of sexually transmitted diseases currently used in practice. Recent data from the HIV Prevention Trials Network Study 052 show ARVs can prevent transmission in HIV discordant couples with 96% efficacy (Anglemyer et al., 2011) and as a direct result of this, the United States Food and Drug Administration (FDA) approved Truvada, an ARV, for pre-exposure prophylaxis (PrEP) for use in healthy but high-risk populations. Due to the high cost of this intervention and the fact that stable, cohabiting couples are often not the case, it has been extensively debated whether this strategy could be efficiently implemented in an under-resourced setting such as Southern Africa. Despite this, HIV acquisition risk declined significantly with increasing ART coverage in a high risk community in Kwa-Zulu Natal, South Africa (Tanser et al., 2013). While the introduction of antiretroviral therapy (ART) has had a dramatic impact on HIV morbidity and mortality (Bor et al., 2013), a vaccine is largely considered to be the

only practical solution to curb the spread of the pandemic. Despite this view, many vaccine trials have been carried out in recent times with little translatable success. The first evidence that vaccination can prevent HIV-1 infection in humans was provided by the RV144 phase 3 clinical trial in Thailand (Rerks-Ngarm et al., 2009), a discovery that rejuvenated the field. The level of efficacy was modest at 31.2% and efforts to understand how to improve the vaccine regimen are on-going. From progress made across all echelons of HIV prevention, it is clear that understanding the mechanisms underlying HIV-1 pathogenesis, with renewed focus on mucosal transmission and the immune system's response to the virus, will allow for improved rational design.

1.2 HIV structure

The overall structure of HIV is illustrated in Figure 1.1A. HIV is classified in the family *Retroviridae* and genus *Lentivirus* and contains two identical copies of single stranded RNA enclosed in a virus capsid, made of multiple copies of the viral protein p24, an antigen commonly used to detect HIV infection. There are three structural genes namely *pol, gag* and *env* which encode the replication enzymes (reverse transcriptase, integrase and protease), the structural proteins of the core and matrix and the viral envelope glycoproteins (gp120 and gp41) respectively. The heterodimeric envelope glycoprotein that allows for attachment and budding to and from the host cell comprises of three gp120 subunits and three gp41 subunits that are non-covalently associated (Figure 1.1B). The sequence of a gp120 monomer is conventionally divided into five conserved constant regions (C1-C5) with five variable regions (V1-V5) shown in Figure 1.1C (Modrow et al., 1987). The crystal structure of gp120 has been elucidated in complex with a number of soluble CD4 receptor and co-receptor binding site directed monoclonal antibodies (mAb) and ligands which has allowed for greater understanding of the interface between the host immune response and the virus itself (Chen et al., 2009; Zhou et al., 2010; Zhou et al., 2007). In the past, to facilitate crystal formation these structures lacked the hyper-variable V1/V2 domain, and in many instances portions of the N-terminal and C-terminal ends and V3 loop. However, the structure of V1/V2 in complex with mAb PG9 has been elucidated (McLellan et al., 2011).

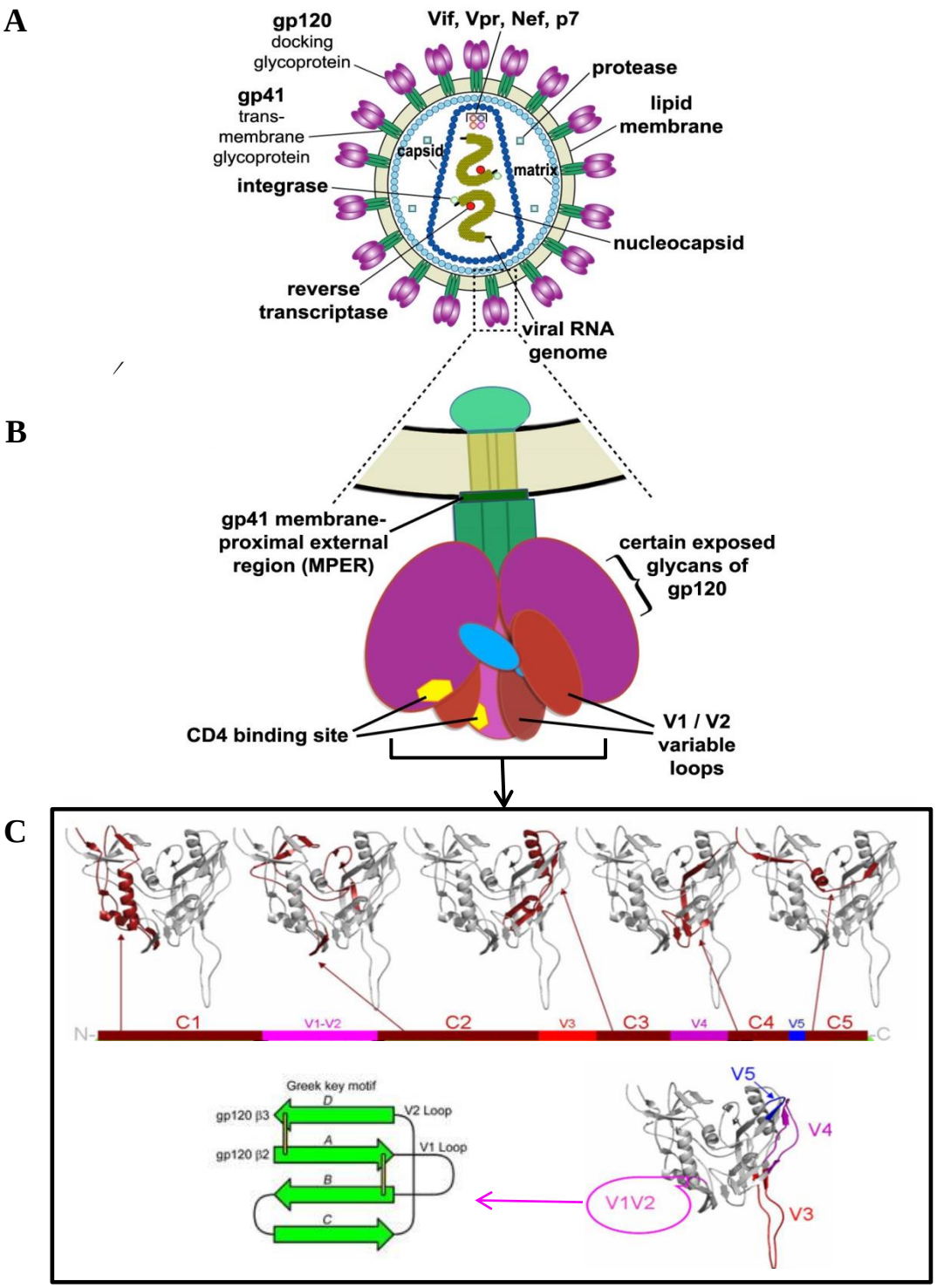


Figure 1.1: Structure of HIV
(A) Schematic representation of the HIV virion with two copies of viral RNA and viral enzymes enclosed in a capsid made of numerous copies of p24. The entire virion core is enclosed in a matrix (p17) and lipid membrane with 3 anchored gp41 subunits and 3 covalently linked gp120 subunits. **(B)** A schematic representation of the glycoprotein envelope spike is shown with V1/V2 loops represented in red, the CD4 binding site is shown in yellow, gp41 shown in green and gp120 subunits in purple (Adapted from (Haynes et al., 2012b). **(C)** A linear schematic of the HIV-1 envelope sequence where the conserved regions C1-C5 are indicated in maroon, and variable sequences V1/V2, V3, V4, and V5 are shown in pink, red, purple, and blue respectively. The secondary structure of the conserved regions (in the first row) and variable loops (second row) are indicated by ribbon diagrams (Adapted from (Wibmer, 2011). The Greek key motif of V1/V2 is shown in green. Strands are shown as arrows and disulphide bonds as yellow boxes (McLellan et al., 2011).

In addition, the uncleaved gp120 trimer at 6Å resolution was recently published (Mao et al., 2013). The envelope structure has great plasticity, a feature accommodated by the variable loops. Among these variable regions, the V1/V2 loop is the most relevant to this study.

V1/V2 structure and function: closer to a successful vaccine

The structure of the V1/V2 region as elucidated by McLellan et al, is shown in Figure 1.1.C and furthermore cryo-electron microscopy illustrated that the V2 loop appears at the tip of the env trimer and is exposed on the envelope (Julien et al., 2013; White et al., 2010). The correlate of protection in the RV144 phase 3 trial in Thailand HIV infection was found to be IgG antibody binding to scaffolded V1/V2 Env proteins (Haynes et al., 2012a), leading to the notion that the design of vaccines to induce high levels of V1/V2 antibodies may enhance vaccine efficacy. The V1/V2 loop is one of the first targets of the early immune response; anti-V1/V2 mAbs have strong neutralising activity against the autologous virus (Gray et al., 2007a; Moore et al., 2008). Indeed, V1/V2 has been described as integral in conformation masking, by partially occluding V3 (Chen et al., 2005). Two potent broadly neutralising mAbs, PG9 and PG16 have been isolated that target the V1/V2 loop (Walker et al., 2009). There is increasing evidence that sequence variation in the V1/V2 loop mediates neutralisation resistance (Moore et al., 2009), viral tropism and cell entry regulation (Cheng-Mayer et al., 1999; Wyatt et al., 1995). The V1/V2 loop is shortened and has fewer N-linked glycosylations in the case of transmitted/founder (T/F) viruses compared to chronic viruses (Derdeyn et al., 2004). Combined, this evidence suggests that not only is this region of gp120 important in the establishment of the transmission bottleneck, but is a common target of the immune system, one that could be exploited in vaccine design. Finally, and most applicable for this study, the putative binding site for the gut homing receptor α4β7 on gp120 is located in the V1/V2 loop (as discussed in Section 1.7).

Potential N-linked glycans (PNGs)

An unusual feature of HIV is the dense glycan shield that encompasses exposed envelope antigens which accounts for more than half of the molecular mass of gp120 (Lasky et al., 1986). The folding of viral glycoproteins, viral transmission, viral escape and host immune response are all significantly

influenced by the presence of these carbohydrates, which are the targets of several potent broadly neutralising antibodies (Scanlan et al., 2007; Walker et al., 2011; Wang et al., 2012). Two major types of protein glycosylation are N-linked and O-linked glycosylation. N-linked glycans are attached to the amide nitrogens of asparagine side chains in the concensus sequences N-X-S/T, where X represents any amino acid residue except proline (Spiro, 2002). O-linked glycans are attached to the hydroxyl group of serine or threonine (Hang and Bertozzi, 2005), however there is no current algorithm to predict these sites. There are approximately 30 sites encoded in the extracellular domain of envelope with two thirds encoded in conserved regions and the remaining third in variable regions (Ochsenbauer et al., 2012; Salazar-Gonzalez et al., 2009). Interestingly, the HIV genome does not include any capability to synthesise carbohydrates; the glycan shield is formed by host enzymes (Scanlan et al., 2007).

Since an ideal vaccine would aim to block the interaction between susceptible host cells and the virus defining the receptors, co-receptors and attachment factors on the host cells and the gp120 trimer is of particular interest.

1.3 Mechanism of HIV binding to receptors and co-receptors

HIV-1 must invade human immune cells to ensure viral spread. Essential to this is attachment to the host cell membrane involving gp41 and gp120 imperative for the recognition of the virus, binding and entry into the target cell. The primary binding action between the host cell and the HIV virion involves CD4 and gp120. In its physiological role, CD4 functions as a receptor for major histocompatibility complex class II molecules during foreign antigen recognition by T cell lymphocytes (Miceli and Parnes, 1993).

During binding, the glycoprotein envelope complex undergoes a structural conformational change rearranging both the gp120 core and the variable loops resulting in exposure of the V3 loop that facilitates binding to chemokine receptors in addition to CD4 (Berger et al., 1999). Chemokine receptors are involved in the homing and recruitment of immune cells to the site of inflammation (Coakley et al., 2005). The most common of these co-receptors for HIV-1 are CCR5 and CXCR4,

however a variety of others have been implicated in the binding of some HIV-1 isolates (Alkhatib and Berger, 2007). The necessity of these chemokine receptors for infection is reflected by the finding that individuals that have a homozygous mutation for CCR5 are resistant to HIV infection (Samson et al., 1996).

Following binding of gp120 to the chemokine co-receptor, gp41 is released from its high energy state and the previously buried fusion peptide springs out towards the host cell membrane (Kilby and Eron, 2003). At this point, gp120 is thought to disassociate from the host cell membrane and gp41 transiently becomes an integral component of the cellular membrane of the host (Doms and Moore, 2000). Gp41 refolds allowing for the release of energy and formation of a fusion pore which ultimately allows the capsid to enter the cytoplasm of the host cell (Doms and Moore, 2000). It is conceivable that there are additional receptors and attachment factors on the host cell that may bind the virus and offer a selective advantage to pathogenesis; one of which will be explored further in this dissertation.

1.4 Clinical immunopathology of HIV

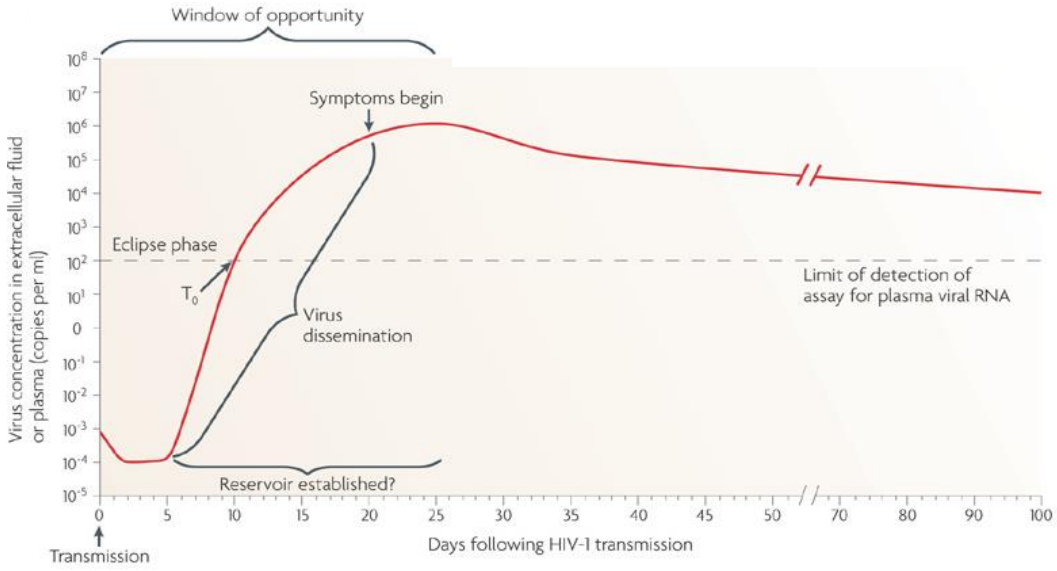


Figure 1.2: Events during acute HIV infection
The period between the time of initial infection and the point of possible viral detection is the eclipse phase. Between 7 and 21 days, the virus disseminates to secondary tissues and reaches peak viremia between 21 and 28 days. Viral concentrations decrease slightly over time and the individual progresses to chronic pathogenesis following 100 days of infection (McMichael et al., 2010).

The pathogenesis of HIV can be simplified as consisting of two parts: an acute phase which lasts approximately 100 days, is designated into Fiebig stages and is characterised by unchecked viral replication and the chronic phase which lasts several years and is characterised by destructive immune activation and weak immune responses (Picker and Watkins, 2005). Events that occur during the acute phase of infection often dictate the kinetics of HIV replication and the rate of disease progression. The eclipse phase is the initial period between the moment when the first cell is infected and virus is first detectable and is thought to be between 7 and 21 days (Lindback et al., 2000). During this clinically silent phase, virus is produced in $CD4^+$ T cells in the mucosa, submucosa, draining lymphatics and, to a modest extent, the gut associated lymphoid tissues (GALT) and systemic tissues (Haase, 2010). Following detectable viremia in the blood plasma (designated T0), levels exponentially increase as a result of the sudden rapid viral expansion in the GALT and peripheral lymphoid tissues reaching a peak at 21-28 days (Brenchley et al., 2004; Ribeiro et al., 2010). By this time, significant and irreversible depletion of mucosal $CD4^+$ T cells has already occurred and some individuals may become symptomatic (McMichael et al., 2010). As the plasma viral load begins to plateau, individuals progress from acute infection to early chronic infection at the end of Fiebig stage V, approximately 100 days following infection. Acute infection is the stage of infection in which a vaccine and other prevention strategies must be most effective.

1.5 HIV transmission

HIV-1 transmission results from viral exposure at mucosal surfaces or from percutaneous inoculation. HIV has been classified phylogenetically into groups M, N and O and P (a new putative designation); each of which represent a separate introduction of SIV from infected great apes into humans (Sharp and Hahn, 2011). Of these groups, M has manifested into the current pandemic, with viral population bottlenecks causing diversification into distinct subtypes (Taylor et al., 2008). HIV-1 clade C is the most common subtype in circulation accounting for 48% of all infections (Stephens, 2012), the most prevalent subtype in the South African pandemic. Despite this level of diversity which could provide

an opportunity for the evolution of altered transmissibility, group M viruses have been remarkably consistent in transmission over time, geography, behaviour and populations (Taylor et al., 2008).

Routes of transmission and the transmission bottleneck

There are multiple routes of HIV transmission including vaginal, rectal, oral, percutaneous, intravenous and in utero. Heterosexual transmission is responsible for approximately 70% of HIV-1 infections worldwide. This is despite the fact that heterosexual transmission is an inefficient process, with an average of only 0.01 transmissions per coital act (Wawer et al., 2005). This risk factor is exponentially increased dependent on the presence of sexually transmitted diseases, male circumcision, socio-economic status, pregnancy, HIV disease stage and route of exposure (El-Sadr et al., 2010; Galvin and Cohen, 2004; Powers et al., 2008) . During early or acute infection, an HIV infected individual in a discordant couple is far more likely to infect their partner than during chronic infection (Brenner et al., 2007; Miller et al., 2010; Powers et al., 2008; Wawer et al., 2005). This may be as a result of the high viral load, a lack of neutralising antibodies that may inactivate circulating virus or clonal expansion of very fit viruses that are particularly suited for the establishment of productive infection (Keele et al., 2008; Richman et al., 2003; Wawer et al., 2005; Wei et al., 2003).

Current observations of HIV transmission suggests that two thresholds need to be overcome for infection to occur: firstly, there may be a threshold of infectious virus needed in genital secretions of the infected partner and secondly there may be a threshold density of susceptible cell targets in the mucosa of the uninfected partner (Iqbal and Kaul, 2008). HIV transmission involves a genetic bottleneck in which a limited number of variants from a diverse quasispecies in the transmitting partner establish productive infection in the newly infected partner (Wolfs et al., 1992; Wolinsky et al., 1992). This definition implies that there are selective pressures acting on these variants, corroborated by the fact that in most cases only 1 virus is able to withstand the genetic bottleneck and productively infect its' host. This virus, known as the transmitted/founder (T/F) virus, can be inferred by using a novel experimental sequencing strategy developed by Keele and colleagues in 2008 based on single genome amplification. It is likely that a number of abortive infections do arise during

transmission but that only one in the case of sexual transmission establishes productive infection (Figure 1.3). It has been determined that approximately 80% of heterosexual subjects are productively infected by a single viral genome irrespective of genetic subtype (Abrahams et al., 2009; Haaland et al., 2009; Keele et al., 2008). Approximately 60% and 40% of men who have sex with men and intravenous drug users respectively, are also productively infected by a single virus. In one case of an injection-drug user, up to 16 T/F viruses were found to cause infection (Bar et al., 2010). Rectal transmission also seems to mitigate the genetic bottleneck that is evident in cervicovaginal transmission (Li et al., 2010). The fact that multiple viruses initiating infection are more prevalent in non-heterosexual transmissions is an indicator of the strength of the mucosa as a barrier to infection.

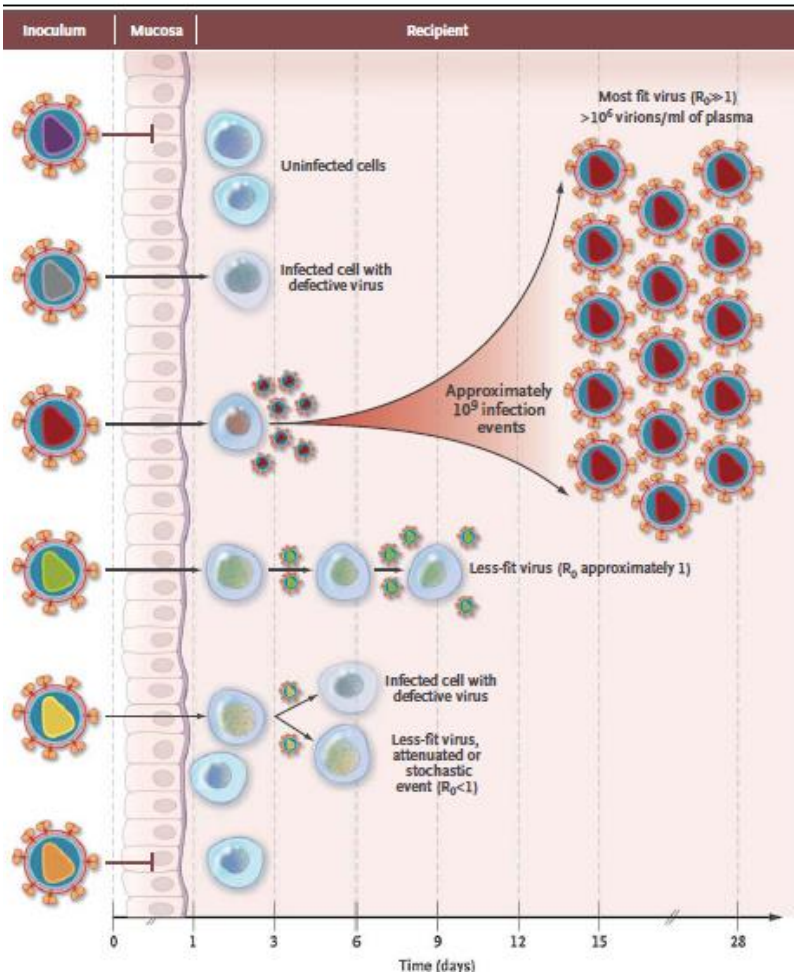


Figure 1.3: Model of HIV infection
While a selection of phenotypically variant viruses are present in the infected fluid and a number of abortive infection events may occur, in most cases only one virus results in productive infection. This may be because other viruses are less fit or fail to come into contact with target cells. During the eclipse phase of infection, viral diversity is thought to be limited (Cohen et al., 2011).

Transmission signatures

Various studies have focused on the determination of a defining phenotype of T/F viruses in order to explain why these viruses are preferentially selected by the transmission bottleneck. Phenotypic identification of transmission signatures has been difficult to correlate with genotypic changes. Transmission of HIV-1 in humans selects for CCR5-tropic viruses, and viruses could be distinguished from CXCR4-tropic viruses genotypically by amino acids located in the V3 region of Env (Jensen and van 't Wout, 2003; Resch et al., 2001). T/F and chronic gp120s have shown similar requirements for elevated levels of CD4 (Alexander et al., 2010; Isaacman-Beck et al., 2009) but differences in CCR5 usage have been determined (Parker et al., 2013). Most recently, Korber and colleagues (Gnanakaran et al., 2011) compared over 7,000 SGA derived *env* gp160 sequences from 275 acutely or chronically infected subtype B subjects and observed statistically robust signatures. The motif with highest statistical significance was in the signal peptide, which may affect Env expression and incorporation of Env into virions (Asmal et al., 2011). How these changes might affect HIV-1 transmission is currently unknown.

T/F viruses from subtypes A, C and D have been determined as having more compact V1-V4 loops with a reduced number of glycans compared to chronic viruses, an observation less clear in subtype B viruses (Chohan et al., 2005; Derdeyn et al., 2004; Ping et al., 2013). DC-SIGN, the C-type lectin expressed on dendritic cells is able to bind virus via oligomannose residues which are expressed to a higher degree on T/F viruses (Geijtenbeek et al., 2000). This process of virus capture may contribute to direct infection of dendritic cells (Muratori et al., 2009), providing a mechanism of immune evasion. It has been suggested that the homing integrin α4β7 binds more efficiently to T/F viruses and that this difference is associated with decreased glycosylation of V1/V2 of gp120 (Nawaz et al., 2011). Studies have shown that these characteristics provide T/F viruses with increased transmission fitness (Chohan et al., 2005; Wu and KewalRamani, 2006).

Envelope proteins from newly transmitted viruses have been shown to retain neutralisation sensitivity to the transmitting partner’s antibodies as well as to CD4-binding-site monoclonal antibodies b12 and

VRC01 (Derdeyn et al., 2004; Wilen et al., 2011). Recent findings show that T/F viruses were more infectious and contained nearly 2 fold more Env incorporated per particle than the chronic controls were recently published (Parrish et al., 2013). Data suggest that T/F viruses have enhanced cell-free infectivity, improved dendritic cell interaction and relative IFN-alpha resistance. Given that HIV-1 transmission is inherently inefficient and likely represents the most vulnerable point in the natural history of HIV-1 infection, identifying unique properties and potential vulnerabilities of T/F viruses remains an important objective for the development of prevention strategies.

1.6 The mucosal immune system: structure and function

The largest component of the mucosal immune system is the gut associated lymphoid tissue (GALT) that spans the gastrointestinal tract from the rectum to the oral cavity and serves to keep at equilibrium the essential functions of the absorption of nutrients and to protect from potential pathogenesis (Mowat and Viney, 1997). Several mechanisms exist within the mucosal immune system to protect from infiltration of pathogens such as a continuous epithelial physical barrier with tight cell junctions, a covering mucus layer, a variety of antimicrobial substances and the maintenance of environmental conditions (for example pH).

Functionally, the mucosal immune system can be divided into inductive and effector sites. Inductive sites are the areas where antigens are collected from the lumen and immune responses initiated including the mesenteric lymph nodes, Peyer's patches and isolated lymphoid follicles. Effector sites are regions where adaptive immune cells exert their immune pressure namely the epithelium and the lamina propria (Brandtzaeg et al., 2008). Anatomically however, lymphocyte populations can be categorised into those present in the epithelium ($CD8^+$ T cells and TϒδT cells) and those in the lamina propria (predominantly $CD4^+$ T helper cells, $CD8^+$ T cells, natural killer cells and B-cells) (Hein, 1999).

The mucosal transmission event

In contrast to the systemic immune system, most of the $CD4^+$ T cells in the mucosal system are $CCR5^+$, activated memory $CD4^+$ T cells. These are preferential targets of HIV and SIV infection (Brenchley et al., 2004) and it is estimated that 60% of all mucosal memory $CD4^+$ T cells are infected by HIV within the first few days following transmission (Mattapallil et al., 2005). HIV-1 traverses several tissue layers in the female genital tract before coming into contact with the appropriate target cells (Figure 1.4 Panel A) such as Langerhans cells or $CD4^+$ T cells in squamous epithelium. $CD4^+$ T cells can also be infected by viruses bound to submucosal dendritic cells. It is believed that a major site for HIV transmission in the female genital tract is the cervix; especially the endocervix and transitional zone which are only covered by a single layer of columnar epithelium (Grivel et al., 2011). This layer is less protective than the stratified epithelium of the vagina (Pudney et al., 2005). The vagina is covered by an exudate which is an effective barrier against many viruses and the upper genital tract is covered in mucus. Soluble factors (such as SDF-1 which binds to CXCR4; defensins, complement proteins and surfactant proteins) found in mucus and the ability of mucus to trap virions may decrease infectivity and slow viral transmission by altering the surface charge of the gp120 timer (Lai et al., 2009).

In the male genital tract, the penile foreskin and urethra harbour target cells (Figure 1.4 Panel B). The outer foreskin is heavily keratinized and is well-protected against pathogenic penetration (Ganor et al., 2010). The inner foreskin and frenulum are covered in a thin layer of keratin and are sites of favourable target cells, such as Langerhans cells, which are presumed to be the first point of contact during penile transmission (Cohen et al., 2011). Virus-cell interactions in the male submucosa are likely to be comparable to the female genital tract with similar targets.

The primary site of transmission in the gastrointestinal tract is the anorectal mucosa and to a lesser extent, the oral mucosa (Lackner et al., 2009). The anus is lined by several layers of stratified squamous epithelium which contains intraepithelial dendritic cells which may facilitate dissemination of virus (Shattock and Moore, 2003). The probability of infection through the rectum and colon is far

higher than the vaginal or penile route. The vulnerability of this tissue lies in the fact that only a single layer of columnar epithelium separates the lumen from the inner layers and may be damaged during sexual intercourse (Nazli et al., 2010). Furthermore, lymphocytes in this tissue are constitutively activated; providing a target population of highly susceptible cells. The oral mucosa does not appear to be a major route of HIV transmission, with the exception of mother-to-child transmission. Despite this, the palatine tonsil is a unique mucosal tissue as it has natural breaks in the barrier to expose underlying lymphocytes to the environment (Lackner et al., 2009).

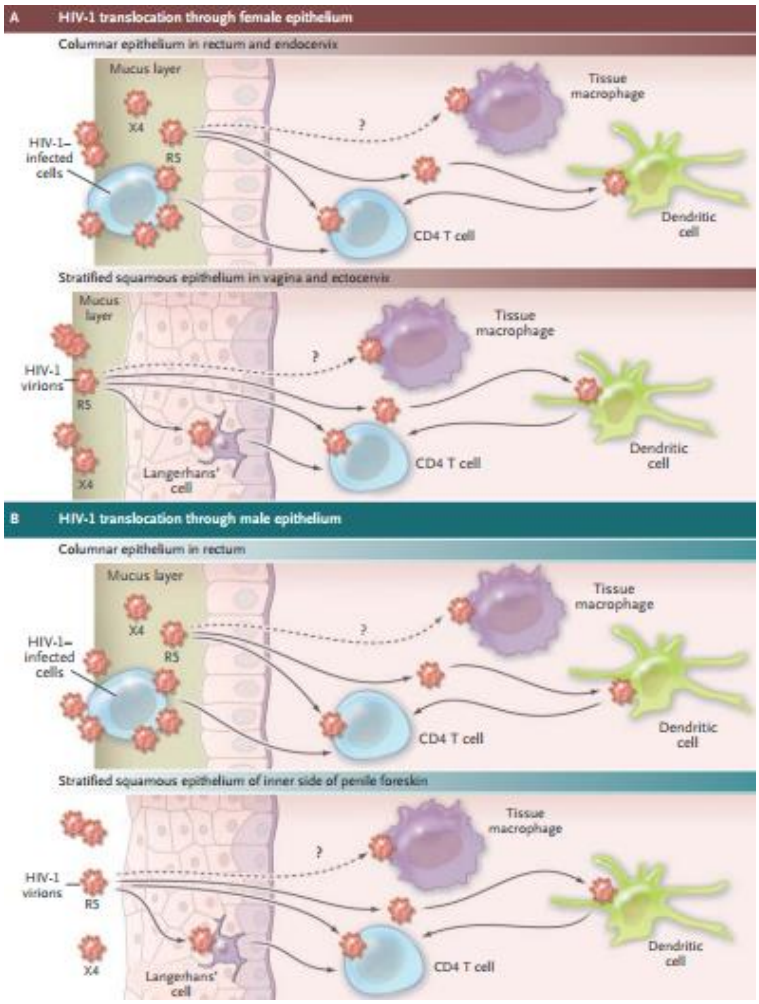


Figure 1.4: HIV transmission through the mucosa
HIV transmission across the **(A)** female and **(B)** male genital tract is shown here. While rectal transmission across thin columnar epithelium and thick mucus layer is comparative between sexes involving dendritic cells, $CD4^+$ T cells and macrophages, transmission across stratified epithelium of the vagina and inner side of the foreskin differ in the presence of a mucus layer in the female genital tract. Dispersed within the stratified squamous epithelium are Langerhans cells (Cohen et al., 2011).

Given the multiple routes of transmission and the distinct histological features of the different tissues involved, it is not surprising that the identity of the initial cells infected with HIV remains elusive. Evidence to date suggests that $CD4^+$ T cells and Langerhans cells are the primary targets (Hladik et al., 2007; van den Berg and Geijtenbeek, 2013) with an implication that dendritic cells may be the initial contact for HIV (Lackner and Veazey, 2007). Macaque studies suggest that mucosal infection may be dependent upon the ability of T/F viruses to infect resting $CD4^+$ effector memory T cells (Haase, 2010). While the virus traverses the mucosal barrier within a matter of hours, the time for dissemination may be between 3 and 6 days. Following this initial step there is rapid dissemination into the draining lymph nodes and systemic circulation along with the establishment of viral reservoirs (Emau et al., 2006). The adjunctive damage to the mucosal tissue following HIV infection causes alterations in intestinal structure and leakage or translocation of gut bacterial products into sterile tissues, leading to further immune activation, increasing HIV replication (Brenchley and Douek, 2008).

Preferential infection of the GALT by HIV

While only 0.01-1% of peripheral $CD4^+$ T cells are productively infected with HIV (Brenchley et al., 2004), in striking contrast 60% of mucosal memory $CD4^+$ T cells becoming infected at the time of peak viremia (Mattapallil et al., 2005). Regardless of the route of transmission, HIV-1 develops a rapid and extensive infection within the GALT which has been described as the principle and initial site of viral replication (Arthos et al., 2008; Brenchley et al., 2006; Lackner et al., 2009). The activated state of the GALT is thought to be as a result of constant antigenic exposure in the junction between the lumen of the gut and the epithelial layers, one of the possible reasons that viral replication is so high in this tissue (Ansari et al., 2011). The mechanism for this preferential cell tropism of HIV-1 for GALT is unknown; however it is hypothesised that the high frequency of $CCR5^+CD4^+$ T cells in GALT provides a rich target environment for HIV-1 replication (Nawaz et al., 2011) and $\alpha4\beta7$ expression on these T cells may facilitate virus infection and replication (Arthos et al., 2008; Kader et al., 2009). In addition the GALT tissue harbours in excess of 40% of all lymphocytes in the body (Hein, 1999).

Homing of T cells to the GALT

As T cells only detect pathogens through direct contact with the MHC-presented antigen, they must traffic to the site of pathogenic exposure. Dendritic cells trap antigens in secondary lymphoid organs and naïve T cells exit the thymus and migrate preferentially to these regions; a process referred to as homing (Butcher and Picker, 1996). The encounter with antigen causes activation and proliferation of effector memory T cell clones that home to sites of inflammation where they come into contact with other cell types to elicit other immune responses. Following antigen clearance, the majority of effector cells die, some remain for long-term protection against repeated exposures to the antigen (Sallusto et al., 1999).

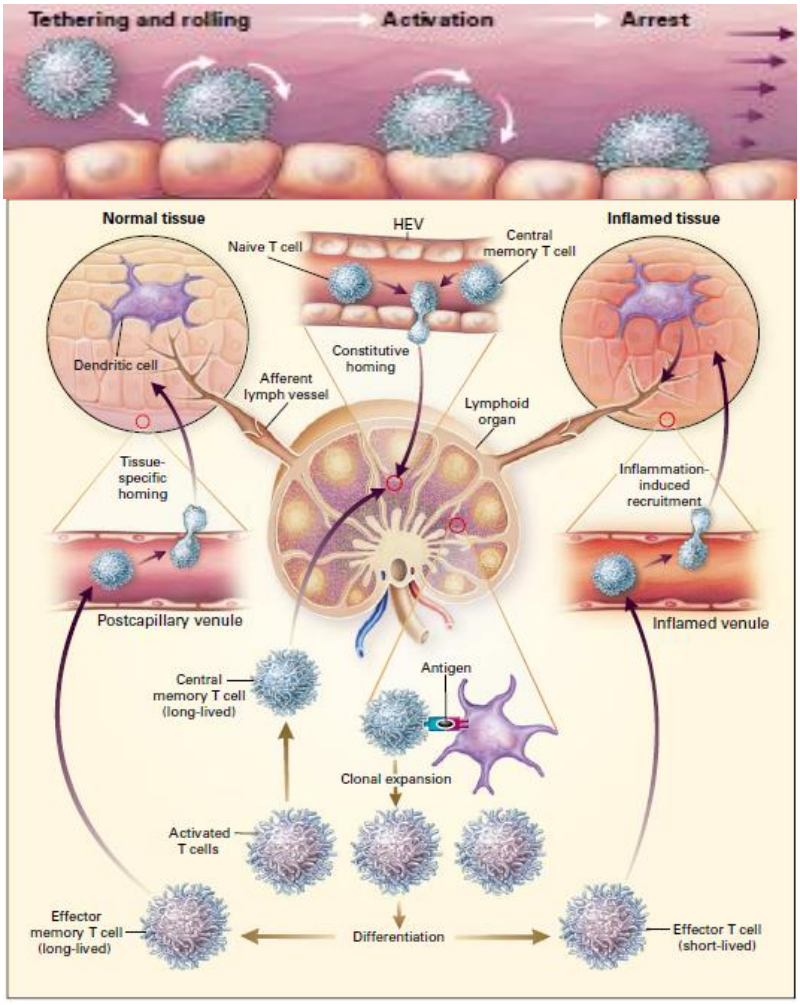


Figure 1.5: Homing of T lymphocytes
Naive T cells home continuously from the blood to lymph nodes and other secondary lymphoid tissues. Homing to lymph nodes occurs in high endothelial venules (HEV), which express molecules for the constitutive recruitment of lymphocytes. On stimulation, T cells proliferate by clonal expansion and differentiate into effector cells, which express receptors that enable them to migrate to sites of inflammation (von Andrian and Mackay, 2000).

In order to leave the circulation and home to various tissues, leukocytes must engage sequential adhesion pathways (Figure 1.5). This sequence involves lymphocyte rolling, chemokine-mediated activation, firm adhesion to the vessel wall and finally transendothelial migration into the tissues. Molecules involved in the cascade include selectins, chemokines and their receptors, integrins and their immunoglobulin superfamily ligands (Berlin et al., 1995; Kim and Broxmeyer, 1999; von Andrian and Mackay, 2000). Specific to the gut, the homing receptors that are relied upon to maintain the levels of lymphocytes are largely the β7 containing integrins α4β7 and αEβ7 and the CCR9 chemokine receptor and its ligand CCL25 (Berlin et al., 1993; Kunkel et al., 2000; Wagner et al., 1996). Of particular interest in this study is the integrin α4β7.

1.7 Integrin α4β7: regulation, structure and function

Integrins are transmembrane cell adhesion receptors that consist of non-covalently associated α and β subunits that mediate cell-extracellular matrix (ECM) and cell-cell interactions (Pribila et al., 2004). Among the adhesion receptors, integrins are unique in the diversity of their ligands and mechanisms of attachment. Vertebrates express 18 α and 8 β subunits that combine to form 24 known integrins (Gorfu et al., 2009). Integrins are responsible for two forms of signalling processes: (i) "Inside-out" signalling involves the transport of proteins to the integrin cytoplasmic domains and multicomplex formation resulting in clustering of integrins and increased avidity for its ligand (Denucci et al., 2009) and (ii) "outside-in" signalling whereby cytoplasmic complexes link the integrin with the actin cytoskeleton and intracellular signalling pathways, allowing for the integrin signalling into the cell. Conformational changes to integrins can elicit cell-signalling events that increase affinity for their ligands, as well as promote cytoskeletal rearrangement and viral internalisation (Stewart and Nemerow, 2007).

Unique amongst the integrins is α4β7. The α4 subunit associates with either the β1 subunit or with β7. The β7 subunit can also pair with the αE subunit, which is expressed on naïve $CD8^+$ T cells and $CD4^+$ T regulatory cells but not naïve $CD4^+$ T cells (Lehmann et al., 2002; Masopust et al., 2006).

Functions of α4β7

While selectins are the primary mediators of tethering and rolling during the homing process, α4β7 uniquely mediates both rolling and firm adhesion and has a specialised structure to do so; with low affinity and high affinity forms triggered for different functions (Yu et al., 2012). α4β7 has been implicated in the formation of secondary lymphoid structures (Mebius et al., 1998), protective immunity against pathogens (Kuklin et al., 2001) and in the progression of gut inflammation; playing a central role in the development of inflammatory bowel disease (IBD) (Feagan et al., 2005), graft versus host disease (GVHD) (Ueha et al., 2007) and diabetes (Iwata et al., 2004). Certain studies have shown that there is a compartmentalised usage of adhesion molecules along the intestine with integrin being of less importance in the large intestine and essential in the small intestine (Bell and Else, 2008).

Ligands of α4β7

The primary ligand for α4β7 is mucosal addressin cell adhesion molecule-1 (MAdCAM-1); an addressin that is expressed primarily on the high endothelial venules of the Peyer's patches, mesenteric lymph nodes and the postcapillary venules of the lamina propria of the gut (Berlin et al., 1993; Streeter et al., 1988). MAdCAM-1 is also expressed in the thymic medulla, tonsils, nasal associated lymphoid tissues, pancreas and brain (Briskin et al., 1997). MAdCAM-1 is highly expressed on the processes of follicular dendritic cells present in germinal centers, mesenteric lymph modes and Peyer's patches and may be involved in the activation of B cells (Carrasco and Batista, 2006). MAdCAM-1 displayed variable expression on the genital tract (Johansson et al., 1999). Its expression is unregulated in areas of inflammation in GALT especially in the case of chronic inflammatory diseases (Kelly and Rank, 1997). In addition to the GALT, it is aberrantly expressed in the eyes, joints, liver and skin during active IBD (Adams and Eksteen, 2006) and upregulated in the genital mucosa following infection with *Chlamydia trachomatis* (Kelly and Rank, 1997). MAdCAM-1 has also been implicated in the homotypic clustering of melanoma cells in malignancy (Leung et al., 2003) and clearly has an extensive and entrenched role in immunity extending beyond the GALT.

MAdCAM-1, vascular cell adhesion molecule -1 (VCAM-1) and fibronectin all bind α4β7 through structurally homologous binding motifs (Jackson, 2002). The minimal binding epitope for MAdCAM-1 is a tripeptide loop Leu-Asp-Thr (LDT), while fibronectin binding requires Leu-Asp-Val (LDV) and VCAM requires Ile-Asp-Ser (IDS). In all natural-ligand binding motifs, the aspartic acid residues are essential to binding, the removal of which abrogates the interaction (Jackson, 2002). However α4β7 binds at a lower affinity to VCAM-1 and fibronectin compared to MAdCAM-1 (Andrew et al., 1994), MAdCAM-1 binds α4β7 by interacting with the metal ion at the metal ion-dependent adhesion site (MIDAS) in the β7 I domain (Sun et al., 2011) allowing for integrin activation and clustering as shown in Figure 1.6. The principal contact site for the natural ligands of α4β7 is present on the α-chain (Zeller et al., 2001).

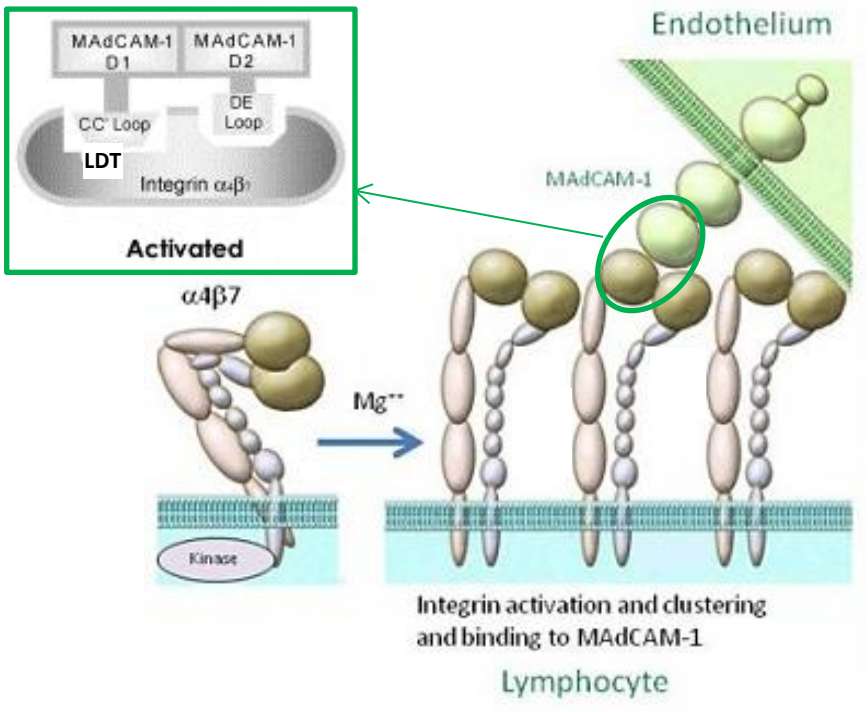


Figure 1.6: Activation and binding of α4β7 to MAdCAM-1
When activated, α4β7 present on the surface of lymphocytes binds to its putative ligand MAdCAM-1in the presence of metal ions. Two sites of contact exist in both domains of MAdCAM-1; one of which is the minimal binding tripeptide motif LDT in the CC' loop of MAdCAM-1 as shown in the green box (adapted from (Papst et al., 2012; Sun et al., 2011).

Regulation of α4β7 expression

Several levels of biological processes regulate α4β7 expression, including transcription, post-transcriptional events, translation in endoplasmic reticulum, dimerization and transport to the cell

surface (Humphries, 2000). Although the majority of circulating T cells express both α4 and β7 subunits, they are mostly in an inactive conformation. As a result of contact with cognate antigen in the Peyer's patches and mesenteric lymph nodes, dendritic cells present in the GALT induce rapid upregulation and induce a higher activation state of gut-homing receptors α4β7 and CCR9 by the action of all-trans retinoic acid (Iwata et al., 2004). $CD103^+$ mucosal dendritic cells in the mesenteric lymph nodes and in the gut lamina propria but not in the skin or spleen express high levels of retinal dehydrogenase (RALDH), an enzyme that is essential for retinoic acid production from Vitamin A (Iwata et al., 2004; Jaensson et al., 2008; Johansson-Lindbom et al., 2005). However, in the absence of dendritic cells, retinoic acid alone is able to upregulate α4β7 and CCR9 expression (Arthos et al., 2008). Retinoic acid increases α4 mRNA levels, while β7 and β1 levels remain constant (DeNucci et al., 2010). There are high amounts of intracellular β7 (in excess of α4 that serve as a reservoir for α4β7 pairing) but not β1 in naïve T cells; therefore overexpressed α4 increases α4β7 expression, without alteration in β1 expression (DeNucci et al., 2010). Another regulation mechanism explains that the *Itg-α4* chain in T cells requires retinoic acid for transcription whereas the *Itg-β7* genes require TGFβ for transcription (Kang et al., 2011). This upregulation has been described on $CD8^+$, $CD4^+$, B cells and natural $CD4^+$ $CD25^+$ $FOXP3^+$ T regulatory cells (Johansson-Lindbom et al., 2005; Mora et al., 2003; Siewert et al., 2007). In contrast, a vitamin D metabolite suppresses retinoic acid induction of α4β7 and CCR9 while increasing the expression of skin homing receptors in T cells (Mora et al., 2008). IL-7, a non-redundant cytokine essential for homeostasis of the T lymphoid compartment, is expressed by stromal cells as well as thymic and intestinal epithelial cells (Watanabe et al., 1995) and is under clinical trials as an immune-reconstitution agent in AIDS (Mackall et al., 2011). Recently, Cimbro and colleagues demonstrated that IL-7 induces expression and functional activation of α4β7 *in vitro* and *in vivo* (Cimbro et al., 2012). While retinoic acid mediated upregulation of α4β7 requires TCR stimulation and occurs on naïve and memory T cells, IL-7 mediated upregulation does not require TCR stimulation, reflecting two mechanisms of α4β7 T cell homing indicative of different physiological states (Cimbro et al., 2012).

α4β7 signalling

Integrin adhesiveness, central to the α4β7-homing function, is regulated by intracellular signalling pathways that are dependent on the cytoplasmic domains of integrins and enforce changes in the integrin conformation, clustering (Yauch et al., 1997), affinity and cell spreading (Krissansen et al., 2006). Small GTP-binding molecules induce binding of α4β7 to its ligand MAdCAM-1 in hierarchical manner involving changes in receptor avidity because of ligand-induced clustering (Zhang et al., 1999). The α4- chain has three LDV motifs which could support cell-binding to $\alpha4\beta7^+$ cells, thus offering an explanation for α4-triggered homotypic cell aggregation (Altevogt et al., 1995).

1.8 α4β7 is an attachment factor for HIV

Adhesion molecules were first implicated in HIV pathogenesis with the discovery that β2 integrins were involved in HIV-induced syncytium formation in T cells and monocytes (Hildreth and Orentas, 1989). Since then, Arthos and colleagues showed that the HIV-1 envelope protein gp120 binds to and signals via the α4β7 integrin on $CD4^+$, $CD8^+$ T cells and NK cells and activates LFA-1 in an α4β7-dependent way (Arthos et al., 2008). This is an important finding because in the tissue where HIV preferentially replicates, the virus' envelope protein interacts directly with a homing receptor integral to $CD4^+$ T cells in the tissue. This mechanism may account, in part, for the GALT being the region of rapid and preferential HIV infection, integral to the dissemination of the virus.

Mechanism of α4β7-gp120 interaction

The principal contact sites for the natural ligands of α4β7 are present on the α4-chain (Zeller et al., 2001). By blocking α4β7 activity with anti-α4, β7 and α4β7 mAbs that target an epitope near MAdCAM-1 (Schiffer et al., 1995), Arthos and colleagues showed that gp120 binds to α4β7 at sites near the target of the natural ligands of the integrin. Present in the V2 loop of gp120 is a tripeptide consensus sequence consisting most frequently of Leu-Asp-Val (LDV) at positions 182-184 (by HXB2 numbering). In addition the aspartic residue in gp120 is conserved in 98% of gp120s present in the Los Alamos HIV database (Arthos et al., 2008; Kwong et al., 2002). The binding of the V2 loop to

α4β7 may facilitate viral entry by lowering the entropic barrier that slows the ligation of envelope spikes to CD4 (Kwong et al., 2002; Zhou et al., 2007). This interaction is highly conserved across subtypes of HIV as well as SIV.

α4β7 co-localises with CCR5 and CD4 on both retinoic acid treated peripheral T cells as well as T cells derived from the gut, with the latter showing increased CD4 clustering (Arthos et al., 2008; Cicala et al., 2009). The position of α4β7 relative to CD4 and CCR5 is shown in Figure 1.7. Using acceptor-photobleaching Forster resonance energy transfer (FRET) and co-precipitation studies, β7 was found to reside within 1.2 nm of CD4 and considering their relative height and the estimated diameter of the gp120 trimer (Kwong et al., 2000), it is likely that the gp120 trimer will encounter α4β7 prior to CD4 (Cicala et al., 2009). HIV binding to α4β7 is independent of CD4 binding, unlike co-receptor (CCR5 and CXCR4) binding (Cicala et al., 2009). α4β7 extends more than 20nM from the cell surface (Chigaev et al., 2003), while comparatively CD4 extends 7nM from the surface (Wu et al., 1997). Unlike the HIV entry receptors CD4 and CCR5, α4β7 is not an absolute requirement for viral replication *in vitro*. This, coupled with its position on the cell surface indicates that α4β7 could serve as an attachment factor for HIV infection and it is likely that it binds gp120 prior to CD4 engagement. This is in line with a structural analysis that places the V1/V2 region of gp120 (contains the tripeptide for α4β7 binding) at the apex of the trimeric spike (Liu et al., 2008; Mao et al., 2013). Different alleles of the *ITGA4* gene, encoding for the α4 subunit of α4β7 have different affinity for natural ligands, anti-α4β7 monoclonal antibodies and gp120. This finding suggests that HIV-1 acquisition, resistance to disease, progression, viremia control and $CD4^+$ T cell depletion may be influenced by *ITGA4* polymorphisms in primate species and humans (Darc et al., 2011).

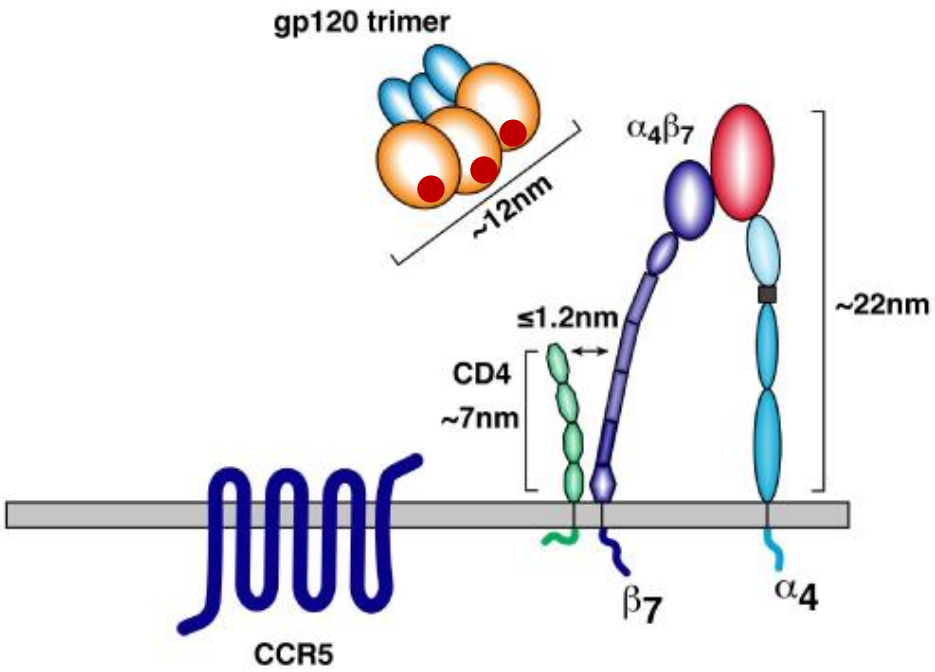


Figure 1.7: Position of α4β7 relative to CD4 and CCR5
The α4β7 integrin extends above the cell surface approximately 3 fold higher than that of CD4. CD4 and α4β7 are very close and can be co-precipitated. V1/V2 loops that contain the α4β7 binding motif are predicted to be on the tip of gp120 and are shown in maroon here (Cicala et al., 2009).

Effects of HIV- α4β7 binding

Apart from the ability to take advantage of the homing function of α4β7 and facilitate dissemination of HIV-1 to the GALT, the effects of binding may be extensive. LFA-1 is an integrin that is linked directly to α4 (Chan et al., 2000) and is essential to immunological synapse formation (Bromley et al., 2001). HIV has been found to commandeer LFA-1 to form virological synapses which facilitate cell-to-cell transmission (Jolly and Sattentau, 2004; Piguet and Sattentau, 2004). Arthos and colleagues illustrated that LFA-1 can be activated by gp120 using a mechanism that is α4β7 dependent and that LFA-1, α4, CD4 and gp120 cluster at the interface of infected and uninfected cells (Arthos et al., 2008). In addition, gp120 is able to drive homotypic clustering and clustering of homing receptors (Birdsall et al., 1997). α4β7 binding increases activation as well as the expression of LFA-1 (Arthos et al., 2008).

α4β7 and HIV-1 transmission

Evidence suggests that the gp120-α4β7 interaction may be especially significant in the earliest stages of HIV infection. In SIV infected macaques, sub-optimally activated memory $CD4^+$ T cells form the founder target population for infection (Li et al., 2005). In humans, non-activated cells in the gut show evidence for productive infection (Mehandru et al., 2007). In these resting cells LFA-1 is mainly inactive and represents a target for gp120-α4β7 mediated interaction (Tardif and Tremblay, 2005). Antigen-specific $\alpha4\beta7^+$ $CD4^+$ T cells are found in the genital mucosa where $CD4^+$ T cells are first infected following sexual intercourse (Hawkins et al., 2000; Kelly et al., 2009). α4β7, through its homing function, is functionally linked to each of the sites involved in acute infection. With the aid of α4β7, HIV is able to gain rapid access to compartments of immunity that have a high number of target cells and as a result, the probability of irreversible infection is greatly increased.

It has been reported that α4β7 high $CD4^+$ T cells are more susceptible to productive infection by HIV-1 than those expressing low levels of the integrin, in part because this subset is enriched for activated $CD4^+$ T cells, and in part because they express high levels of CCR5 and low levels of CXCR4 (Cicala et al., 2009). This phenotype extends to blood, rectum, colon and genital mucosa of the female reproductive tract (cervical cytobrush) (Cicala et al., 2009; Kelly et al., 2009). It has been reported that during the acute phase of SIV infection, α4β7 high $CD4^+$T cells are preferentially infected and that $\alpha4\beta7^+$ T cells are depleted in lamina propria and rectal lymphoid nodes (Kader et al., 2009; Vajdy et al., 2000). In humans, circulating $\alpha4\beta7^+$ $CD4^+$ T cells are preferentially depleted following acute HIV infection (Krzysiek et al., 2001). Prior to the onset of viral infection (except in the case of other inflammatory disease of sexually transmitted disease and other compounding factors), the fully activated $CD4^+$ T cells are in the minority (Zhang et al., 2004b) and with most of these expressing α4β7, it is likely that α4β7 plays an important role in the establishment of infection. The genital mucosa does not contain organised immune-inductive sites but relies upon $\alpha4\beta7^+$ T cells to traffic from organised inductive sites including the Peyer's patches (Mestecky and Fultz, 1999) and it is possible that the initial and most relevant site for the gp120-α4β7 interaction is the genital mucosa in the initial days following transmission. As a result of sexual transmission being a highly inefficient

process, any advantage would be of benefit for HIV replication. The impact of the α4β7-gp120 interaction varies for different viral isolates, even for clones within the same individual at similar periods of pathogenesis (Arthos et al., 2008). This suggests that the stronger the affinity for α4β7, the higher the transmission fitness of the virus. Three early–transmitting gp120 monomers were found to have higher α4β7 reactivity than their matched chronic counterparts. To this end, Nawaz and colleagues showed that at the time of mucosal transmission selection for T/F viruses that have reduced glycosylation correlates with an increase in α4β7 reactivity and that this may be a transmission signature exploited for therapies (Nawaz et al., 2011). This effect appears to be linked to the change in conformation of V1/V2 and C3/V4 rather than steric occlusion (Nawaz et al., 2011). In the case of Chlamydia infection, antigen-specific $\alpha4\beta7^{+}$ T cells increase in number in the female genital tract homing to the GALT, suggesting that isolates with the ability to engage α4β7 are more likely to come into contact with rich target cells (Hawkins et al., 2000; Kelly et al., 2009; Kelly and Rank, 1997). Therefore, bottlenecks created as a result of sexually transmitted co-infections may determine α4β7 reactivity at transmission.

In contrast, Parrish and colleagues have described that *in vitro* anti-α4β7 mAbs had no inhibitory effect on viral replication and therefore no selective advantage offered by α4β7 during HIV pathogenesis with the exception of SF162, a subtype B virus (Parrish et al., 2012).While this result is pertinent, because much of gp120-α4β7 interaction hypothesis is based on binding studies, the chronic and T/F viruses studied were not matched and were of a small number with only six T/F and six chronic viruses examined. Furthermore concentrations of inhibitory mAbs used and determined as saturating in α4β7-binding assays increased HIV replication in many cases as a result of homotypic clustering triggered by α4β7-mAb binding.

Despite this controversy, SIV infected rhesus macaques treatment with anti- α4β7 mAbs, displayed a decreased viral load and reduction of viremia and proviral DNA load in the GALT (Ansari et al., 2011). This proof-of-concept suggests a role for α4β7 in HIV transmission. During acute SIV and

HIV infection α4β7 has been found to be upregulated on $CD4^+$ T cells as well as NK cells (Cummins et al., 2001; Reeves et al., 2010), suggesting a role for the integrin in dissemination.

1.9 α4β7 defines a subset of T lymphocytes highly permissive to infection

α4β7 is expressed on naïve T and B cells at a low level (Erle et al., 1994). It is also expressed on NK cells, monocytes, dendritic cells, macrophages and eosinophils. The expression of this integrin is however enhanced on IgA expressing plasma cells and on memory gut-homing $CD4^+$ subsets (Schweighoffer et al., 1993). α4β7 denotes a subset of CCR5 high memory $CD4^+$ T cells that are strongly reactive for Ki67, a marker of cellular metabolism and activation (Cicala et al., 2009) evident and present in peripheral blood as well as in rectum, colon, female reproductive tract (Cicala et al., 2009) and cerebrospinal fluid (Kivisakk et al., 2006).

It has been reported that α4β7 high $CD4^+$ T cells are more susceptible to productive infection by HIV than those expressing low levels of the integrin, in part because this subset is enriched for activated $CD4^+$ T cells, and in part because they express high levels of CCR5 and low levels of CXCR4 (Cicala et al., 2009). Highly α4β7-expressing $CD4^+$ T cells with a resting memory phenotype serve as the dominant target of SIV infection during acute infection (Kader et al., 2009).

A delicate balance exists in the intestinal tract to maintain protection of the tissues without inducing autoimmune inflammation. This balance is disrupted in the GALT during HIV infection. At least two $CD4^+$ T cell subsets have been found to maintain this balance and their disruption may be the cause of microbial translocation during HIV pathogenesis. These subsets, Th17 (pro-inflammatory) and T regulatory cells (anti-inflammatory) shown in Figure 1.8, are present in the GALT and genital mucosa, are regulated by retinoic acid and express α4β7, and as a result, are of interest in order to understand the initial dissemination of the virus to sites distal to the genital mucosa.

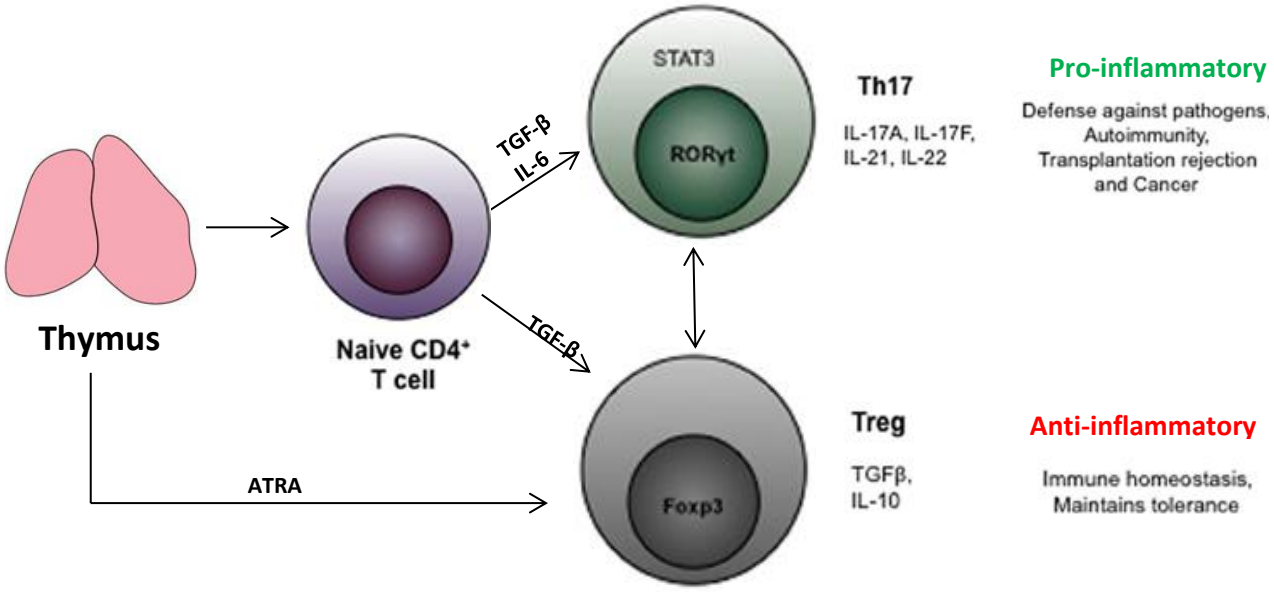


Figure 1.8: Development of Th17 and Treg CD4$^+$ T cells
Polarisation of naïve T cells to Th17 CD4$^+$ T cells occurs via stimulation by TGF-β and IL-6 and Treg CD4$^+$ T cells by TGF-β. Treg cells can also be induced by ATRA. Treg and Th17 cells exist in equilibrium with anti-inflammatory Treg cells supressing Th17 pro-inflammatory ability. Adapted from (Sethi et al., 2013).

Th17 and T regulatory cells in HIV and immunity

A) T helper 17 cells

Th17 cells are CD4$^+$ T cells that play a critical role in the maintenance of mucosal surfaces including enterocyte homeostasis and defence against extracellular bacteria and fungus. Functioning in the mediation of inflammation (Bettelli et al., 2006) and the development of autoimmunity, these cells have also been linked with the recruitment of neutrophils and macrophages to the infected tissues and have been found in peripheral circulation as well as the genital mucosa (McKinnon et al., 2011). Th17 cells produce IL-17 (a marker that typically identifies them in most phenotypic studies), IL-21 and IL-22, but not interferon gamma or IL-4 (Steinman, 2007) They express the lineage specific transcription factor RORϒt (Ivanov et al., 2006) and most IL-17$^+$ T cells are CCR6$^+$ (Brucklacher-Waldert et al., 2009; Singh et al., 2008). CCR6 is a marker necessary for cell migration into the Peyer's patches of the small intestine (Wang et al., 2009a). A number of other markers for the Th17 subset have been identified but are not well-characterised including CD161 (Cosmi et al., 2008) and CD152 (Brucklacher-Waldert et al., 2009). Th17 cells differentiate in the presence of TGF-β and IL-6 (Mangan et al., 2006; Yang et al., 2008) and the impact of retinoic acid on their phenotype is controversial and requires further investigation.

Th17 cells are enriched in the lamina propria of the GALT (Ivanov et al., 2008). Interestingly, CCR5 and α4β7 expression is substantially increased on Th17 cells present in the cervix compared to those in the blood (McKinnon et al., 2011). The high expression of IL-17 has been shown in multiple sclerosis, rheumatoid arthritis and IBDs, most of which are currently treated by blockade of α4β7 (Tzartos et al., 2008). Currently little is known about the homing of Th17 cells to sites of inflammation in the intestinal tissues and the kinetics of α4β7 expression on these cells under conditions that favour the Th17 phenotype (Gorfu et al., 2009). Indeed cells that express IL-17 but not β7 exhibit a 50% decrease in trafficking to the GALT (Gorfu et al., 2009). Th17 $CD4^+$ T cells require IL-7 for survival and expansion (Liu et al., 2010), an interleukin that has recently been described to upregulate α4β7 (Cimbro et al., 2012). It has been reported that most Th17 $CD4^+$ T cells express α4β7 (Ansari et al., 2011) and another study, suggested that $\alpha4\beta7^+$ are mostly Th17 $CD4^+$ T cells (Kader et al., 2009).

Multiple susceptibility factors for HIV are expressed in tandem on Th17 cervical T cells. These cells are depleted preferentially in cervical tissue and bind gp120 and as such, this population has been suggested as a founder target group for HIV transmission (McKinnon et al., 2011). Evidence suggests that Th17 cells are the most prominently depleted $CD4^+$ T cell subset following HIV/SIV infection (Brenchley et al., 2008). Besides being highly susceptible to infection, the loss of Th17 cells provides a direct link between $CD4^+$ depletion and immune dysfunction, as the major function for IL-17 is the strengthening of mucosal epithelial tight junctions (Blaschitz and Raffatellu, 2010). In nonhuman primates, depletion of Th17 cells following SIV infection was associated with loss of *Salmonella typhimurium* control leading to system leakage of bacterial components (Raffatellu et al., 2008). Th17 cells are lost in the gastrointestinal tract of HIV individuals (Brenchley and Douek, 2008) but another study has shown that HIV infected individuals have higher IL-17 levels than healthy individuals (Maek et al., 2007). The loss of Th17 cells has been found to be predictive of systemic and sustained T cell activation (Favre et al., 2009).

Th17 cells themselves are highly susceptible to HIV infection and express high levels of CCR5 (El Hed et al., 2010). It has been shown that SIV replication in the infected rhesus macaque is limited by the size of the pre-existing Th17 cell compartment: those that had a higher representation pre-infection (higher Treg: Th17 ratio) had a lower viral set point (Hartigan-O'Connor et al., 2012) and are therefore correlated with protection against replication acutely and for months following infection. In chronically infected HIV individuals the frequency of T cells is rapidly decreased and a higher frequency of viral DNA is found in Th17 cells (Gosselin et al., 2010). Indeed these cells may be restored via antiretroviral treatment if therapy is started at the time of infection (Alvarez et al., 2012; Macal et al., 2008). Since α4β7 is an attachment factor for HIV, preferential depletion of Th17 cells during HIV infection has been described as a result of α4β7 high expression on these cells (Kader et al., 2009). However, CCR6 being a marker of Th17 cells and critical for migration into the small intestine has been found to denote a subset that is highly permissive to HIV infection regardless of the expression of α4β7 (Monteiro et al., 2011). A recent study showed that Th17 $CCR6^+$ T cells expressed higher levels of α4β7 and bound gp120 more extensively (Alvarez et al., 2013). Understanding the mechanism of Th17 cell depletion during HIV and the phenotype of those that express susceptibility markers such as α4β7, is imperative for the development of future therapies for restoration of immune function in HIV infected individuals.

B) T regulatory T cells

T regulatory cells (Tregs) serve to attenuate immune responses and remain in homeostasis with pro-inflammatory Th17 cells by efficiently suppressing their effector functions (Crome et al., 2010). While the phenotypic markers expressed by Th17 cells are well defined, those of Treg cells are not. The most comprehensive definition of the phenotype currently used is CD25 high $FOXP3^+$ CD127neg/low $CD4^+$ T cells, although the correlation between CD25 and FOXP3 in humans is far less prominent than in mice (Liu et al., 2006; Valmori et al., 2006). Recent findings however indicate that some Treg cells can produce IL-17 and IL-17 negative Treg cells can convert into IL-17 producing cells (Auvert et al., 2005; Deknuydt et al., 2009). $FOXP3^+$ T cells that are produced in the thymus from T cell progenitors are known as natural Treg cell. However FOXP3+ T cells known as

inducible Tregs are also found in the GALT, the development of which is mediated by ATRA secreted by GALT-dendritic cells in synergy with TGF-β (Sun et al., 2007). The phenotypic marker used to differentiate the latter from the former is the presence of Helios (Thornton et al., 2010), but it is controversial and therefore these two groups are not really differentiated in humans but rather in mice for passive immunotherapy of inflammatory diseases. This peripheral induction is accompanied by an increase in α4β7 expression to illicit enhanced homing to the GALT where inflammatory homeostasis is required (Benson et al., 2007). It is unknown whether Treg cells use conventional homing receptors such as α4β7 to migrate to the gut like effector cells (Gorfu et al., 2009). Gut homing deficient Treg cells are still able to elicit suppressive functionality in the GALT (Denning et al., 2005), this suggests that there are alternate routes of trafficking Treg cells to this region. About 50% of $FOXP3^+$ Treg cells express L-selectin which can bind MAdCAM-1 (Huehn et al., 2005). As a result the mesenteric lymph nodes may be a site where β7-deficient cells accumulate and inhibit the initial priming of pathogenic T helper cells.

A significant drop in $FOXP3^+$ Treg cells has been noted during SIV and HIV infected individuals (Andersson et al., 2005; Estes et al., 2006). However, a number of more recent studies reported an increase in Treg T cell number following HIV infection has also been described in lymph nodes and duodenal tissue (Li et al., 2011a; Shaw et al., 2011) as well as a slight elevation in blood (Kinter et al., 2004). Stage of infection is an important factor as well with studies reporting either a lower or higher Treg proportion during acute infection and the majority of studies showing an increase in Treg frequencies in chronic infection regardless of phenotyping strategy used (Chevalier and Weiss, 2013). Reports indicate that frequencies of Treg cells in the GALT are significantly higher than in the blood following HIV infection suggesting, homing of these cells (Rios et al., 2012). While agreement on the correct phenotypic markers for Treg cells is controversial, studies have shown that FOXP3 alone is increased and is a marker for HIV disease progression (Rallon et al., 2009; Suchard et al., 2010; Zhang et al., 2008). It is also possible that it is the relative balance of Treg and Th17 subsets, rather than either alone that drive HIV pathogenesis (Kanwar et al., 2010). Treg cells in HIV and SIV may decrease chronic immune activation which may slow disease progression (Kinter et al., 2004) or they

may inhibit antiviral responses, thereby increasing disease progression (Nilsson et al., 2006). While the latter is more widely accepted in the literature, discriminating clearly between which of these is occurring *in vivo* has been fraught with contradictions.

Several studies have shown *in vitro* that Treg cells are highly susceptible to HIV infection (Chase et al., 2008; Moreno-Fernandez et al., 2009; Oswald-Richter et al., 2004). Moreover, HIV-1 infection in Treg cells has been described as increasing α4β7 expression (Ji and Cloyd, 2009) and survival (Nilsson et al., 2006). Studies have shown that antiretroviral therapy can restore Treg cell levels to that of a healthy control, similarly to Th17 cells (Epple et al., 2006; Montes et al., 2011).

Taken together, these data suggest that Th17 and Treg $CD4^+$ T cells may have a role in viral dissemination to secondary tissues following transmission.

1.10 α4β7 as a therapeutic and vaccine target

Integrins are attractive drug targets for interfering with cell proliferation, migration and tissue localisation. By modulating essential elements of the homing of naïve T cells or dendritic cells, clinicians might prevent, attenuate, or enhance immune responses to new antigens, such as allografts or vaccines. Because this treatment would not interfere with the responses of memory T cells, it should not be globally immunosuppressive and therefore may be a suitable approach (von Andrian and Mackay, 2000).

One therapeutic option to prevent inflammatory disease is perturbing the de-adhesion of integrins by mutation, shown to decrease migration of leukocytes to inflamed tissue (Park et al., 2007; Semmrich et al., 2005). Natalizumab is a humanized antibody that is directed against α4β7 and α4β1integrins and has shown efficacy for the treatment of multiple sclerosis and Crohn's disease (Ghosh et al., 2003; von Andrian and Engelhardt, 2003). Interestingly, Natalizumab was removed from the market for a period because several patients who had received additional immunosuppressive therapy developed progressive multifocal leukoencephalopathy as a result of the reactivation of the John Cunningham (JC) virus (Langer-Gould et al., 2005), bringing into question the validity of this

treatment for other diseases. Such therapeutic targeting should take into account that there are multiple roles of adhesion molecules in the immune system and blocking them is likely to be a double-edged sword that may have effects ranging from lymphocyte costimulation, polarisation and survival. Humanised Act-1 (Vedolizumab) demonstrated efficacy in clinical trials for ulcerative colitis and Crohn's disease (Feagan et al., 2005) and is the first antagonist showing a reduction of viral load in SIV infected macaques (Ansari et al., 2011). Interestingly, novel vaccine targets are also being investigated such as a strategy that targets ALDH1a2, a retinoic acid producing enzyme in GALT dendritic cells. Silencing the enzyme resulted in a decreased expression of α4β7 and redirected gp140 specific immune responses from the gut to the genital tract, allowing for α4β7-low immune response (Zhu et al., 2013).

$\alpha4\beta7^+$ T cells have been implicated in a number of therapeutic and vaccine strategies; the level of $\alpha4\beta7^+$ T cell expression in the blood has been investigated as a marker for tracking $CD4^+$ T cell loss in the intestine as a result of SIV infection (Wang et al., 2009b). The failure of the STEP trial (a randomised vaccine trial of a T cell based vaccine aimed at limiting HIV replication) was attributed in part to the expansion of $\alpha4\beta7^+$ $CD4^+$ T cells as a result of the use of Adenovirus type 5 vector for vaccination (Chakupurakal et al., 2010). The only HIV vaccine to show some level of protection, the RV144 Thai trial, showed antibodies to V1/V2 as a correlate of protection – the gp120 region in which α4β7 binding motif is present. Furthermore, Rolland and colleagues showed that vaccine efficacy directed at the tripeptide α4β7 binding motif was 78% if viruses were mismatched to the vaccine at position 181(Rolland et al., 2012). Indeed there are naturally occurring V2 antibodies in HIV infection that have been found to target the α4β7 epitope during HIV infection including 697-D, of which the epitope includes two residues of the LDV/I motif (Gorny et al., 1994; Gorny et al., 2012; Nakamura et al., 2012). A recent publication also showed that a mutation in the α4β7 binding motif dramatically increased the binding of several mAbs directed at V1/V2 (Mayr et al., 2013). It is becoming clear that blocking α4β7, its gp120 binding site or inducing antibodies specific for this region in a vaccine may be a viable option as a means to fight the scourge of HIV.

Aims and objectives

Overall aim of this study

The aim of this study is to investigate the role of integrin α4β7 at various stages of HIV pathogenesis, to define α4β7-expressing $CD4^+$ T lymphocytes with focus on Th17 and Treg subsets and to determine how α4β7 expression affects permissiveness of these subsets to acute HIV infection.

Rationale for this study

The role of integrin α4β7 in HIV pathogenesis has been controversial, with gp120 monomeric proteins derived from early transmitting viruses showing increased binding to α4β7 (Nawaz et al., 2011) . However, in a study by Parrish et al using infectious replicative virus, this observation could not be repeated. Using 6 unmatched T/F and chronic IMCs pairs they found no difference in their affinity for α4β7 (Parrish et al., 2012). While the binding of whole virions to the integrin is far more biologically relevant, determining the role of α4β7 in HIV pathogenesis in this manner with matched viruses longitudinally over time from several individuals is needed to adequately address this question.

In addition, although α4β7 has been shown to define a subset of CCR5 high $CD4^+$ T cells that are highly activated (Cicala et al., 2009), the relevance of this subset in the genital mucosa at the point of transmission and the GALT and the link between these two regions has not been fully elucidated. Two subsets that exist in equilibrium in these regions of mucosa have been determined as α4β7-expressing; Th17 and Treg $CD4^+$ T cells (Ji and Cloyd, 2009; Kader et al., 2009; McKinnon et al., 2011). While the Th17 subset has been extensively linked with the integrin in a pro-inflammatory role, Treg $CD4^+$ T cells have not been as widely investigated in this manner despite the fact that these cells are regulated by ATRA produced by GALT-dendritic cells, similar to the upregulation of α4β7 (Kang et al., 2007). Establishing expression levels of the integrin on these subsets and how permissive they are to acute HIV infection, may provide a possible route of viral dissemination from the site of transmission. The elucidation of a clear role for α4β7 throughout the course of infection and viral

dissemination may allow for the integrin to be exploited either as a preventative target or as a therapy for HIV-infected patients to reduce the damaging effects of immune activation.

Specific Aim 1:

To examine the nature of the binding interaction between α4β7 and infective HIV virus, the dependence on α4β7 for viral replication at different stages of HIV-1 pathogenesis and factors responsible for differences between time points and individuals.

Specific Aim 2:

To determine the phenotype of α4β7 expressing $CD4^+$ subsets under GALT conditions (mirrored via ATRA treatment) with specific focus on the Th17 and Treg subsets and examine how permissive they are to acute HIV infection.

CHAPTER 2

THE DEPENDENCE ON $\alpha_4\beta_7$ FOR VIRAL REPLICATION CHANGES OVER THE COURSE OF HIV SUBTYPE C INFECTION

2.1 Introduction

Regardless of the route of transmission, HIV establishes a rapid infection in the GALT (Arthos et al., 2008). In the case of sexual transmission, the genital mucosa does not contain organized immune-inductive sites but relies upon integrin α4β7 to traffic from organised inductive sites including the Peyer's patches, and mesenteric lymph nodes (Mestecky and Fultz, 1999). α4β7 was identified as an attachment factor for the envelope protein of HIV, gp120 (Arthos et al., 2008). Thus it is possible that the initial and most relevant site for the gp120-α4β7 interaction is the genital mucosa in the initial days following transmission.

The natural ligands of α4β7 MAdCAM-1, VCAM-1 and fibronectin, arranged in order of preference for α4β7, all bind through structurally homologous binding motifs (Jackson, 2002). The minimal binding epitope for MAdCAM-1 is a tripeptide loop Leu-Asp-Thr (LDT), while fibronectin binding requires Leu-Asp-Val (LDV) and VCAM, Ile-Asp-Ser (IDS). In all natural-ligand binding motifs, the aspartic acid residues are essential to binding, the removal of which abrogates the interaction (Jackson, 2002). The importance of α4β7 in HIV transmission and pathogenesis is reflected in the ability of α4β7 to bind HIV and that the aspartic acid imperative for α4β7 binding on V1/V2 of gp120 is conserved in 98% of HIV-1 isolates present in the Los Alamos HIV sequence database (Arthos et al., 2008). Of all the sequences in this database, 72% have a LDI/V motif however, this motif is less conserved in subtype C at 42% than in other subtypes (B: 76%, D: 79%, AE: 74%, A: 86%, AG: 58%). Sequences from South Africa also show a decreased conservation of the LDI/V motif (47%) as compared to 85% of sequences from Malawi and 81% of sequences from Zambia. Although a highly resolved HIV trimer structure has not yet been elucidated, V1/V2 appears in a position that would allow for adequate binding in line with the trimer structure from Joseph Sodroski and colleagues as well as the V1/V2 structure on the gp120 monomer as determined by McKellan et al in 2011 (Mao et al., 2013; McLellan et al., 2011).

The ability of $\alpha4\beta7^+$ T cells to home to secondary lymphoid tissues and the GALT (Berlin et al., 1993; von Andrian and Mackay, 2000) coupled with their presence at the site of sexual transmission (Kelly

et al., 2009; McKinnon et al., 2011), and their co-expression with multiple HIV susceptibility markers (Cicala et al., 2009) suggests a level of selective advantage offered by the integrin for viral replication. Data from SIV infected rhesus macaques show that following treatment with anti-α4β7 mAbs there is a decreased viral load and proviral DNA load in the GALT (Ansari et al., 2011) corroborating a role for α4β7 in the facilitation of replication.

In a study by Nawaz, et al, gp120s representative of early-transmitting viruses were found to have a higher affinity for α4β7 than those of chronic viruses with fewer glycans in V1/V2 and C3/V4 associated with higher α4β7 reactivity. However, sample sizes were very small (3 in total) (Nawaz et al., 2011). Contrary to observations by Nawaz and colleagues, the effect of α4β7 on replication has been deemed to remain unaffected by shorter glycosylated V1/V2 loops in matched early (1-6 months) and chronic (ranging between 24-47 months) viruses from eight individuals (Etemad et al., 2013). Although intriguing, these data generalise early viral time points, do not consider T/F viruses and defined chronic viruses at different time points across individuals. Furthermore, a study by Parrish and colleagues investigated six T/F and four chronic infectious molecular clones to determine dependence on α4β7 for viral replication with the finding that there was no preferential affinity of the T/F replicative virus for the integrin (Parrish et al., 2012). Once again, sample sizes were small and were unmatched. Unmatched samples are not ideal for this comparison as they allow for the contribution of possible confounding factors. As such, the role of α4β7 longitudinally during the course of HIV infection and the factors that may define α4β7 reactivity have not been clearly elucidated. Whether the affinity for α4β7 can be defined as a transmission signature remains controversial.

In this study, we used infectious viruses from 11 participants from the CAPRISA cohort, matched over a range of time points (T/F, 1/2, 6, 12 and 39 months post infection) to determine a role for α4β7 in HIV pathogenesis. We postulated that the immune environment at the area of HIV transmission and tripeptide binding motif changes may influence the affinity of the T/F viruses for α4β7. These factors, as well as length and glycan density of the variable loops in gp120, distance of glycans from the

conserved aspartic acid in the tripeptide α4β7-binding motif and position of glycans were taken into consideration while assessing the effect of α4β7 on HIV replication and more specifically, the importance of α4β7 in the transmission event.

2.2. Materials and Methods

2.2.1. Ethical Considerations and Study Population

Human samples were previously obtained from HIV women enrolled into the CAPRISA 002 Acute HIV Infection Cohort, established in 2004 (van Loggerenberg et al., 2008). Participants were derived from a prospective cohort of 245 high-risk HIV negative African women of which 62 became HIV infected and from whom stored material is available. Clinical sample collection was done at enrolment, weekly for 3 weeks, fortnightly until 3 months, monthly until 12 months, and quarterly thereafter. It is a well characterised cohort covering a large demographic area and despite the mobility of this sex worker cohort, the retention rate after 2 years was 86.1% (van Loggerenberg et al., 2008). Data including CD4 count, viral load and STI status were supplied by CAPRISA having been obtained as described previously (Mlisana et al., 2012; van Loggerenberg et al., 2008). Written and informed consent was obtained from all participants and the parent study received ethical approval from the University of KwaZulu-Natal (Ethics number: E013/04), University of Cape Town and University of Witwatersrand (Ethics Number: M040202) which is renewed annually. Ethics for my study was obtained from the Human Research Ethics Committee (HREC) of the University of the Witwatersrand M120221, the certificate of which follows in Appendix A.

2.2.2. Isolation and stimulation of $CD4^+$ T lymphocytes from whole blood

$CD4^+$ T cells were isolated from whole blood of healthy donors to a purity of 99%, obtained from the South Africa National Blood Service (SANBS), using RosetteSep Human $CD4^+$ T cell Enrichment Cocktail (Stem Cell Technologies, Canada) as per the manufacturer's protocol. ATRA (Sigma-Aldrich, Missouri) was used to induce a gut-homing phenotype on the isolated $CD4^+$ T cells in culture with the most physiologically relevant and optimum concentration determined as 10 μM following the

titration of ATRA in line with various studies (Arthos et al., 2008; Iwata et al., 2004; Kang et al., 2011). In addition, 50 ng/ml OKT3 (anti-CD3 antibody) (eBioscience, California) and 20 U/ml IL-2 (Roche Applied Sciences, Germany) were added to the $CD4^+$ lymphocytes in RPMI media with 20% FBS and cultured for six days at 37˚C at 5% CO2 (Arthos et al., 2008). A control for upregulation of α4β7 was performed under the same IL-2 and OKT3 conditions but no ATRA was added.

2.2.3. Flow cytometry surface staining

The purity of the $CD4^+$ T cell isolation and the upregulation of α4β7 was routinely confirmed by flow cytometry by staining with optimally titrated amounts of CD3-ECD (Beckham Coulter, France), CD4-Qdot 605 (Invitrogen, Carlsbad, CA), Anti-Human CD49d (Integrin alpha 4) PE (eBioscience, CA) and Anti- Human/Mouse Integrin β7 FITC (eBioscience, CA) and Aqua Fluorescent Reactive Dye (Invitrogen, Molecular Probes, Carlsbad) was used to define cell viability. Fluorochromes were diluted in a 1% BSA/ PBS buffer up to a staining volume of 50 µl. 50 µl of cells were then added to the staining mixture and incubated in the dark for 30 minutes at 4 ˚C. After a series of washes with the staining buffer, live/dead staining with Aqua Fluorescent Reactive Dye was done as above. Following staining, cells were fixed in 0.1% paraformalydehyde in PBS. Figure 2.1 shows the gating strategy for $\alpha4\beta7^+$ $CD4^+$ T lymphocytes. Fluorescence minus one controls (FMOs) shown in the Appendix (Figure B4) were used to define gates and compensation controls for multicolour staining were made fresh for each experiment were made using beads. Any direct comparisons were carried out on the same day, with voltages and resulting MFIs monitored over time by the use of peak 3 beads (BD). Donors were designated responders and non-responders to the ATRA treatment based on the increase or no effect, respectively, of the ATRA treatment on the $\alpha4\beta7^+$ population of lymphocytes. 25% of the donors responded to ATRA treatment and only these donors were used in further experiments. Acquiring of all samples was performed on a FACSAria (BD Biosciences). All data analysis was done on FlowJo X software (TreeStar Ashland, OR).

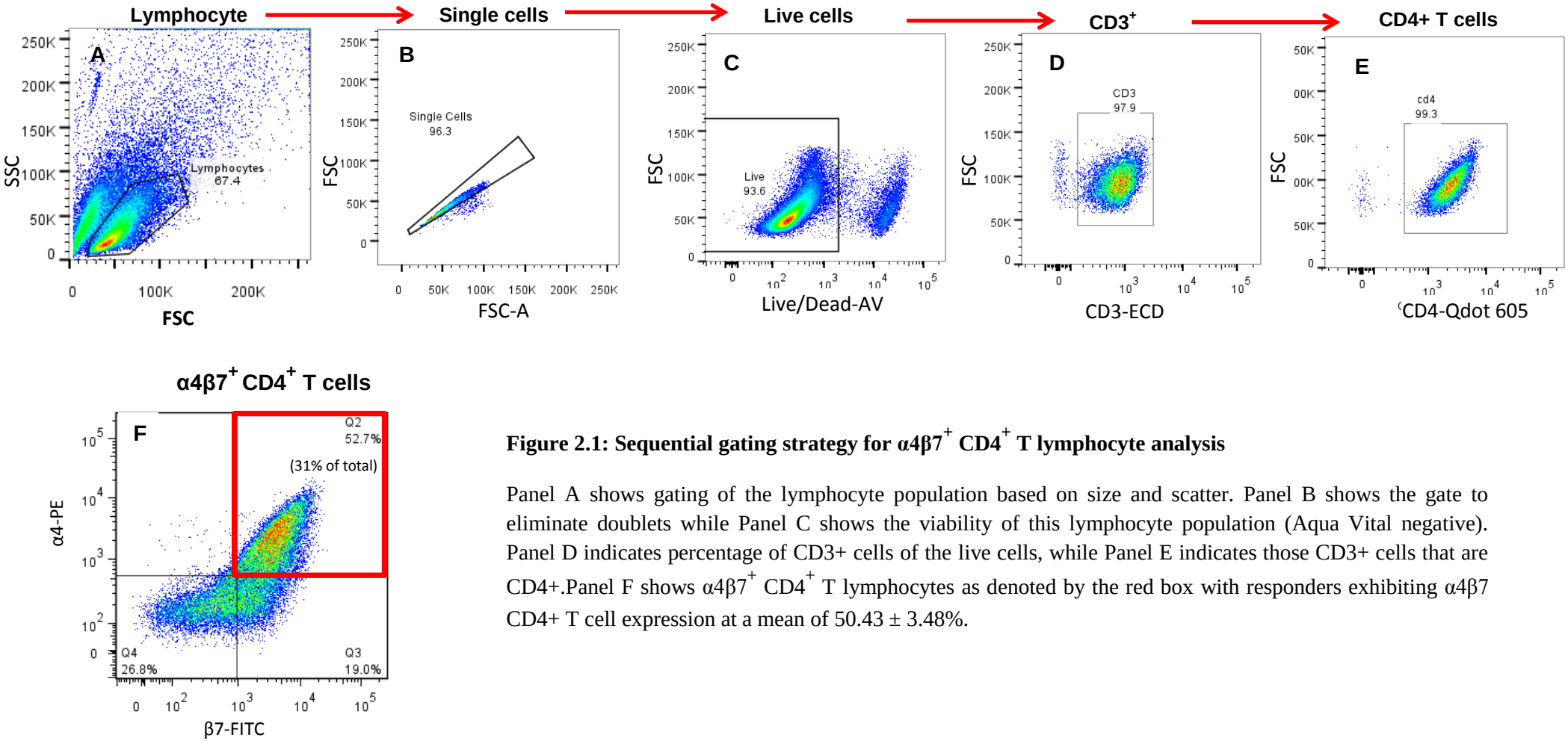


Figure 2.1: Sequential gating strategy for α4β7^{+} CD4^{+} T lymphocyte analysis

Panel A shows gating of the lymphocyte population based on size and scatter. Panel B shows the gate to eliminate doublets while Panel C shows the viability of this lymphocyte population (Aqua Vital negative). Panel D indicates percentage of CD3+ cells of the live cells, while Panel E indicates those CD3+ cells that are CD4+.Panel F shows α4β7^{+} CD4^{+} T lymphocytes as denoted by the red box with responders exhibiting α4β7 CD4+ T cell expression at a mean of 50.43 ± 3.48%.

2.2.4 Production of infectious envelope clones and infectious molecular clones

Replication competent viruses, defined here as infectious envelope clones or IECs, were constructed using *env* genes from 11 CAPRISA 002 participants designated as rapid or intermediate progressors and whether the participants go on to produce broadly cross neutralising antibodies (Table B1 in the Appendix). These IECs included T/F viruses, acute (1- 3 months) and chronic infection (6-39 months) virus clones shown in Table 2.1. A detailed description of all IECs in this study is indicated in Table B2 of the Appendix. These *env* genes were generated by single genome amplification (SGA) for previous studies and were ligated into a pcDNA3.0 vector by others in the laboratory (Keele et al., 2008). The *env* gene sequences were amplified by PCR from these plasmids in this study with the reaction mixture as per Table B3 and Figure B1 of the Appendix. The presence of the correct size band was then confirmed by electrophoresis as shown in Figure 2.2B, following which the PCR products were purified by means of the Qiagen QIAquick PCR Purification Kit (Qiagen, Valencia, CA) as per the manufacturer's protocol.

Table 2.1: IECs from 11 CAPRISA 002 participants used in this study

	T/F	Acute 1-3 mo	Chronic 6 mo	Chronic 12 mo	Chronic 39 mo
CAP 8	1			1	2
CAP 88	1	1	1	6	3
CAP 177			2	3	2
CAP 200	1		1	3	2
CAP 206	1	1	2	2	5
CAP 210	1				
CAP 225	1				
CAP 239	1				
CAP 244		1			2
CAP 255		1			3
CAP 256	1	1			7
TOTAL	**8**	**5**	**6**	**15**	**24**

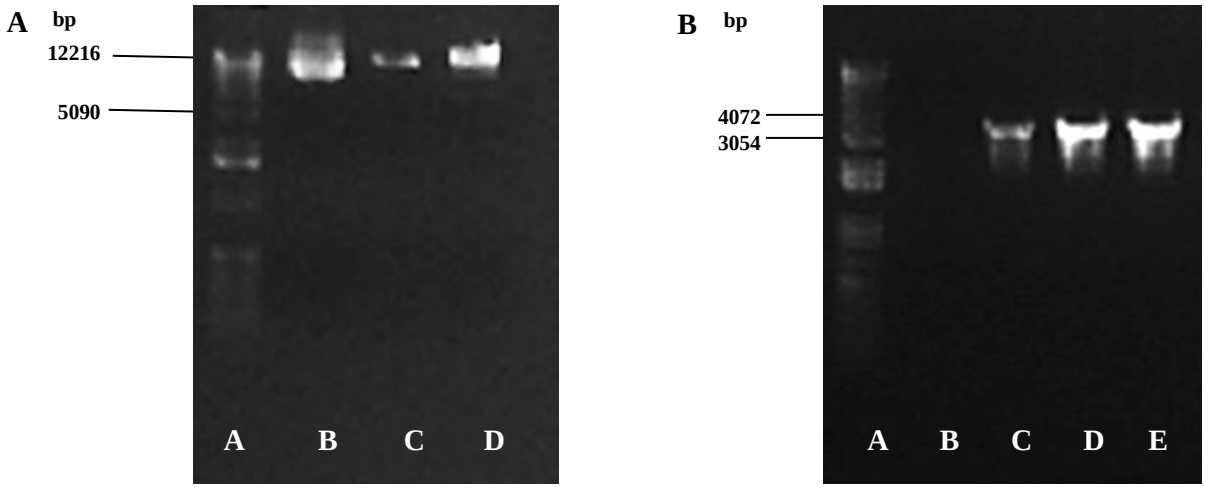


Figure 2.2: Confirmation of components for infectious envelope clones
A: Confirmation of pHIVΔenvBstEIInef-hisD plasmid cleavage by BstEII with lane A DNA Molecular Weight Marker X; lane B: uncut plasmid (> 12.2 kbp), with C and D exhibiting a linearilised plasmid (12 kbp) by EcoRI (positive control) and BstEII respectively. **B:** Confirmation of *env* gene PCR with lane A: DNA Molecular Weight Marker X; lane B: shows the negative PCR control and C-E show the *env* genes of clones x, y and z from 39 month post infection of individual CAP255 (3.5 kbp).

Replicating viruses were prepared from specific envelope clones as described (Lu et al., 2004). The vector pHIVΔenvBstEllnef-hisD (a gift from Dr Kuritzkes), was constructed as described elsewhere (Lu et al., 2004). The plasmid was linearised in this study by means of the restriction enzyme BstEII with the reaction mixture as indicated in Table B4 in the Appendix. The reaction mixture of 100 µl was then reacted at 60˚C for 1 hour and compared with the migration of EcoRI digested plasmid as a positive control in gel electrophoresis (shown in Figure 2.2A) as it, in addition to BstEII, only cleaves the plasmid once. The PCR product was purified by means of the Qiagen Qiaex II Gel Extraction Kit as per the manufacturer's protocol for desalting and concentrating DNA solutions (Qiagen, Valencia, CA). The digested backbone (750 ng) and the amplified *env* DNA (300 ng) were co-transfected with X-tremeGENE Transfection Reagent (Roche, Basel) into HEK293T cells seeded at 3 x 10^5 cells/ml in 12 well plates.

In addition two infectious molecular clones or IMCs, matched to the IEC T/Fs from participants CAP210 and CAP239 and gifted to us by Prof Carolyn Williamson's laboratory at UCT, were transfected in the same manner as IECs in our laboratory. They comprised a single plasmid and were

transfected at a concentration of 600 ng. Following incubation at 37 ˚C and 5% CO2 for 72 hours, the media was changed to fresh DMEM with 10% FBS. After a week of incubation in HEK293T cells, 1 ml of the viral supernatant was spinoculated for 60 minutes at 1200g, 30˚C with activated $CD8^+$ depleted PBMCs at a concentration of $5x10^6$ cells/ml. The following day, this centrifugation step was repeated. IMCs were grown in the same way, to ensure high titer of virus. A week later, an in-house p24 antigen ELISA was used to determine if there was viral growth over this time period. Viruses that displayed growth were then expanded into T25 flasks containing freshly isolated CD8 depleted PBMCs at 2 $x10^6$ cells/ml and following several more days of incubation harvested 3 times. During harvesting, the viral supernatant was removed from the flask and filtered with a 0.45 nm micropore filter and aliquoted for freezing at -70˚C.

In-house p24 ELISA

The ELISA was performed as per protocol in (Moore et al., 1990) involving a chemiluminescent readout on the Wallac Victor II luminometer (PerkinElmer, Waltham, MA). The assay is a twin sandwich enzyme-linked immunosorbent assay (ELISA) whereby the p24 antigen is captured from a detergent lysate of virions onto a polyclonal antibody absorbed to a solid phase. Bound p24 is detected with an alkaline phosphatase-conjugated anti-p24 monoclonal antibody while the primary coating mAb was D7320 Sheep anti-HIV p24 gag, affinity purified antibody (Aalto Bio reagents, Dublin) at 4µg/ml in a carbonate/bicarbonate buffer (Sigma-Aldrich, San Diego, CA). D7320 is a mixture of three sheep polyclonal antibodies raised against HIV peptides (specifically amino acids 173-188; 226-237; 283-297). The p24 standard (Recombinant HIV-1-p24, Aalto Bio reagents) was diluted 1:31.25 in 1% Empigen/TBS and serially diluted 1:4 in 1% Empigen/TBS and along with the harvested viruses were incubated in the blocked plate for 3 hours. A negative control well was included (1% Empigen/TBS) as well as p24 positive controls.

TCID50 Assay

Both IECs and IMCs were titrated in luciferase reporter cell line JC53bl-13 (also known as TZM-bl) obtained from the NIH AIDS Research and Reference Reagent Program. Luciferase activity was measured in relative luminescence units (RLU) which is directly proportional to the number of

infectious virus particles present in the initial inoculum over a range of values. The TCID50 (50% tissue culture infectious dose) of each virus stock was determined in a single-cycle infection assay (2-day incubation) in TZM-bl cells as developed by David Montefiori in 2004. This endpoint dilution assay quantifies the amount of virus required to infect 50% of host cells. The assay was read by a Wallac Victor II luminometer (PerkinElmer, Waltham, MA).

2.2.5 Flow cytometry based α4β7-virus binding assays

HEK293T cells were transfected with α4 and β7 encoding plasmids (Origene, Rockville, MD) with X-tremeGENE and incubated at 37˚C with 5% CO_2 for two days. The transfected cells were removed gently with 1mM EDTA/ PBS, following which the co-expression of α4β7 was determined by flow cytometry by staining with anti-human CD49d (Integrin alpha 4) PE (eBioscience, CA) and anti-human/mouse integrin β7 FITC (eBioscience, CA). The gating strategy was done as shown in Figure 2.3. Two forms of the binding assay were used; a (A) competition based assay and a (B) direct binding assay.

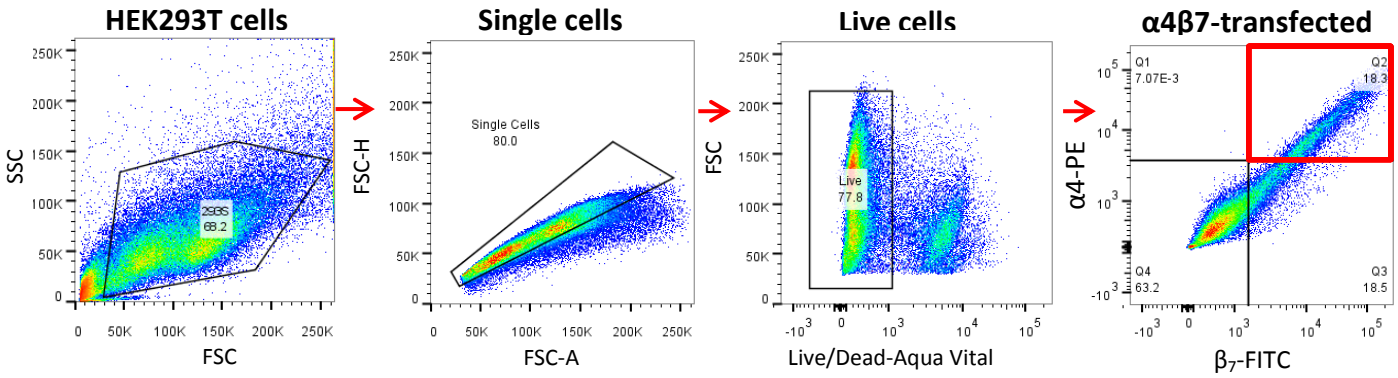


Figure 2.3: Gating strategy for an α4β7 –dependent virus binding competition assay
HEK293T cells transfected with α4β7 were gated to eliminate doublets, dead cells and distinguish $\alpha4\beta7^{+}$ cells outlined by the red box (second, third and fourth panels from the left respectively), gating for which was determined by FMO controls.

(A) Competition α4β7 binding assay

Based on the gp120- binding assays performed by Nawaz and colleagues in 2010, HEK293T α4β7-transfected cells were incubated in the presence or absence with Act-1 (targets the α4β7 dimer) obtained from the NIH AIDS Research and Reference Reagent Program or HP2/1 (Beckman Coulter,

France) which targets the α4 subunit only, for 15 minutes and with or without IECs for 25 minutes. CAP88.1.00.17.5a (T/F) and CAP88.12.2F (12 month p.i) IECs were used in this assay as were matched CAP210 and CAP239 T/F IECs and IMCs. Cells were then stained with α4-PE and β7-FITC mAbs (eBioscience, CA) and a live/dead viability stain namely Aqua Fluorescent Reactive Dye (Invitrogen, Molecular Probes, Carlsbad). Percentage binding to α4β7 is defined as the percentage difference between the median fluorescence intensity (MFI) of PE and FITC of α4β7-transfected cells without an inhibitory mAb or virus and the MFI α4β7-transfected cells with an inhibitory mAb or virus represented in a formula below:

$$\% \text{ binding to } \alpha4\beta7 = \frac{(\text{MFI } \beta7\text{-FITC of untreated sample}) - (\text{MFI } \beta7\text{-FITC HP2/1 OR Act-1 OR virus samples})}{(\text{MFI } \beta7\text{-FITC of untreated sample})}$$

(B) Direct α4β7 binding assay

α4β7-expressing, HEK293T cells were incubated with or without HP2/1 or Act-1 for 15 minutes at 4˚C and incubated with or without virus for 25 minutes without washing following mAb incubation. Viruses used were CAP88.1.00.17.5a (T/F) and CAP88.12.2F (12 month p.i) IECs were used in this assay as were matched CAP210 and CAP239 T/F IECs and IMCs. P24- FITC (KC57, Beckman Coulter) was incubated with the virus-cell complex for 30 minutes at 4 ˚C as well as those cells without any virus added. Percentage p24 positive cells gated on total HEK293T cells were compared to those incubated with virus and those without as a representation of affinity of the virus for α4β7.

2.2.6 α4β7 mediated virus capture inhibition assay

In order to observe the effect that α4β7 has on the replication of HIV-1, an α4β7 mediated virus capture inhibition assay was performed. The assay was designed on the principle that the masking of the α4β7 integrin by a monoclonal antibody has been shown to have an inhibitory effect on viral replication (Cicala et al., 2009).

Titration of α4β7-inhibiting mAbs HP2/1 and Act-1

Both HP2/1 and Act-1 mAb concentrations were optimised by titration in the α4β7-mediated virus capture inhibition assay, noting the concentration at which effectiveness of blocking viral replication

was highest. In addition, a flow cytometry based competition assay was designed where 100 µl of ATRA treated $CD4^+$ T cells seeded at $1x10^5$ cells/ml were incubated for 1 hour with the mAb using a range of concentrations (0.2 pM- 30nM for Act-1 and 2.2 pM – 30nM for HP2/1). An α4-PE labelled antibody was then used to stain the cells in 1% BSA/PBS. The difference between the MFI of α4-PE on $CD4^+$ T lymphocytes without Act-1 or HP2/1 and the MFI of $CD4^+$ T lymphocytes incubated with HP2/1 and Act-1 was used to represent the effect of the inhibitory mAbs as change in MFI.

α4β7-inhibition assay

25 µl ATRA-treated $CD4^+$ T lymphocytes from a responder individual at 4×10^6 cells/ml were either incubated with 25 µl of media (virus control), 10 µg/ml anti-CD4 monoclonal antibody, 275 pM inhibiting monoclonal antibody HP2/1 (targeting α4 subunit) or 2.2 pM Act-1 (targets α4β7) for an hour at 37˚C at 5% CO_2. 100 µl of titrated IECs and IMCs (25 $TCID_{50}$) were incubated for 2 hours with the cells in a 96-well plate, followed by two washes with fresh IL-2 media (5% IL-2 at 200 U/ml, 20% FBS and RPMI media) at 1200g for 5 minutes at 25˚C. Culture supernatant was harvested into 1.25% Empigen/TBS solution and stored at 4˚C, without disturbing cells for a period of 10 days every 2 days with replacement of fresh IL-2 media. P24 levels were assessed by the p24 ELISA as previously described. These experiments were repeated in triplicate.

2.2.7 Statistics

Paired data with only two groups were assessed by paired t test or Wilcoxon matched pairs signed rank test and in groups with three or more sets by the repeated measures one way ANOVA with pairwise comparisons adjusted for multiple comparisons using Tukey's method. Unpaired data were assessed for significance by the Mann-Whitney test or the one way ANOVA and the Fisher exact test was used to determine the significance of categorical groupings. While Spearman's correlation was use to assess linear correlations, multiple regression analysis was performed to assess the significance multiple factors (STATA 12). Observations of $p < 0.05$ were defined as significant. Statistics and graphs were done with GraphPad Prism 5 and STATA 12.

2.3 Results

2.3.1 Titration of α4β7 inhibitory mAbs HP2/1 and Act-1

In order to assess the saturating concentrations of the inhibitory mAbs Act-1 and HP2/1 to block α4β7, a flow-based competition assay between α4-PE and Act-1 or HP2/1 (Figure 2.4A and B i and ii) was used. The basis of a competition assay is that by binding to the same epitope or epitopes in very close proximity, the initial antibody will block the binding of the second fluorescently labelled antibody; resulting in a diminished signal (in this case PE-MFI) which is expressed as a change in PE-MFI (Δ MFI of α4-PE). A range of concentrations were tested (0.2 pM- 30nM for Act-1 and 2.2 pM – 30nM for HP2/1) , The highest concentration tested for both HP2/1 and Act-1was the most effective at competing with the α4 (CD49d) –PE mAb and decreased in their capacity to block α4β7 in a dose dependent manner (Figure 2.4 A and B). It was necessary to determine if the saturating concentration of the inhibitory mAbs determined in a binding assay translated to a decrease in viral replication. ATRA responder PBMCs were incubated with a range of HP2/1 or Act-1 mAb concentrations (0.2 pM- 30nM for Act-1 and 2.2 pM – 30nM for HP2/1) and the CAP88.1.00.17.5A T/F IEC. Monitoring replication over 10 days by p24 ELISA revealed that the ability of Act-1 and HP2/1 to block replication by blocking α4β7 at 30 nM does not correlate with the level of saturation. 30 nM of Act-1 and HP2/1 enhanced infection. Act-1 has been found to decrease heterotypic binding but increase homotypic binding at higher concentrations (Zeller et al., 2001) which may explain why it facilitates increased replication. Instead, a reproducible concentration at which replication can be inhibited was determined as 275 pM for HP2/1 and 2.2 pM for Act-1 (shown in Figure 2.4 C I and ii respectively). CAP88 T/F virus was used as a control virus as a result of showing a high affinity for α4β7 in a previous study (Nawaz, et al., 2011). The effect of HP2/1 and Act-1 on viral replication is only partial, while complete inhibition of viral replication results from treatment with an anti-CD4 mAb, corroborating that α4β7 is not essential for replication but rather offers a means for enhanced infection. In this study, the titration of the inhibiting mAbs was crucial to adequately determine the range of dependence on α4β7 for replication in further experiments.

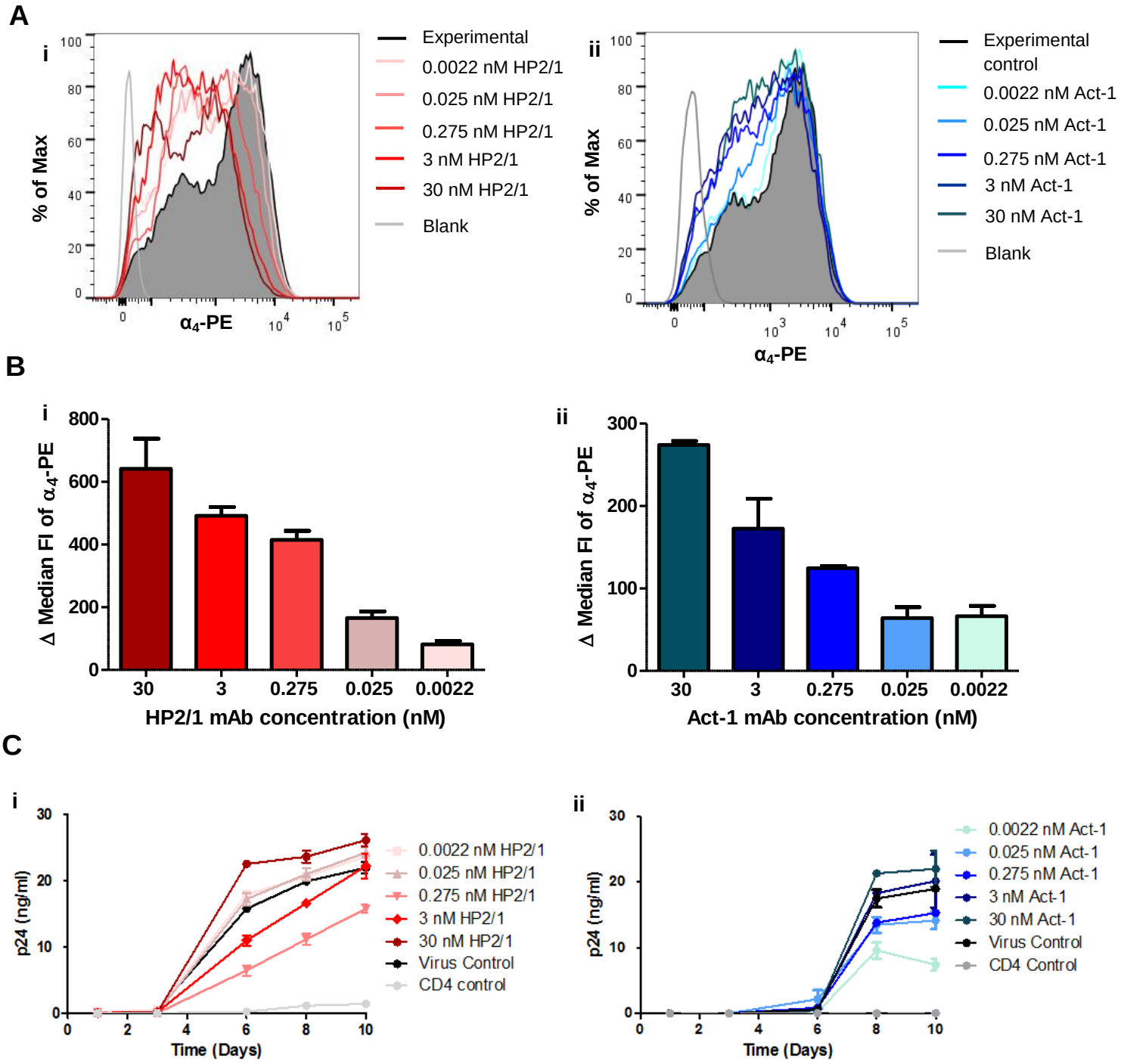


Figure 2.4: Titration of α4β7 inhibitors for maximal inhibition of viral replication
(A) A decreasing shift in PE florescence relative to the experimental control (ATRA treated $CD4^+$ T cells alone shown in black); is indicated in a dose-dependent manner between 0.0022-30 nM (i) HP2/1 and (ii) Act-1concentrations (red and blue graduations respectively). All samples were gated on single live $CD4^+$ T lymphocytes. **(B)** Change in median fluorescent intensity (MFI) with the 30 nM being the most saturating concentration for blocking of the integrin by both mAbs. These results are representative of four repeated experiments, with the bars showing the mean and error bars, the SEM. **(C)** The same ATRA activated responders were infected with a T/F virus CAP88.1.00.17-5A and incubated with (i) HP2/1and (ii) Act-1 between 0.0022-30 nM (red and blue graduations respectively) and monitored over 10 days by p24-ELISA. The virus control (no inhibitory mAb) is shown in black and the infectivity control shown in grey. The optimum concentration for viral inhibition by HP2/1 is 0.275 nM and Act-1 is 2.2 pM, while the viral replication is significantly upregulated at 30 nM in both cases. These results are representative of five independent experiments using 3 donors,with error bars indicating the SEM and points, the mean of three replicates.

2.3.2. α4β7 binds functional virus

To verify that the infectious envelope viruses in this study bound α4β7, two binding assays were set up: a direct binding assay and a competition based assay similar to that described by Nawaz and colleagues. In both cases transfection of the HEK293T cells with α4 and β7 plasmids was confirmed by co-expression of α4-PE and β7-FITC in the experimental control (transfected with α4β7 as compared to the transfection control (was not transfected with α4β7) shown in red and black in Figure 2.5. A negative virus control (was not transfected with α4β7 but was incubated with virus) was included in both assays to ascertain whether virus bound HEK293T cells in the absence of the α4β7 integrin and to assess whether virus incubation increased non-specific florescence of the sample indicated in blue (Figure 2.5).

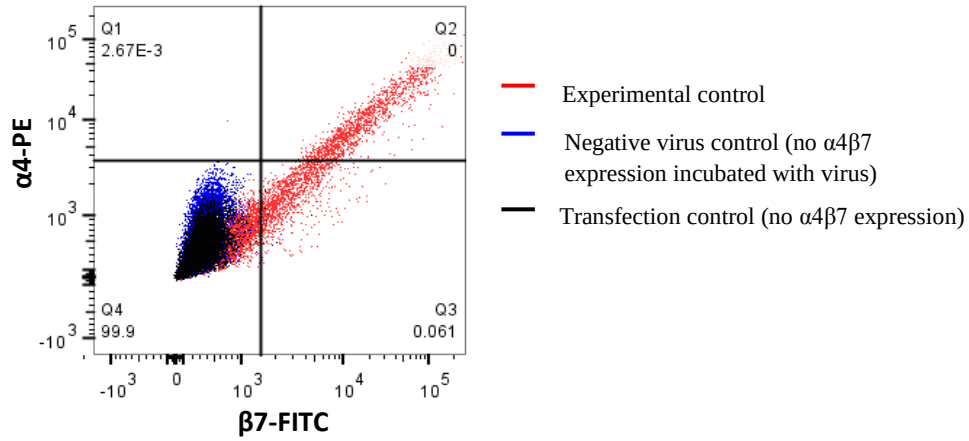


Figure 2.5: Expression of α4β7 on the HEK293T cell line
Gating was determined by both FMO controls and using a transfection control (HEK293T cells alone) indicated in black and a negative virus control (HEK293T cells incubated with virus) shown in blue. A positive experimental control (α4β7-transfected HEK293T cells incubated with virus) is shown in red.

The direct binding assay was performed by incubating α4β7-transfected and untransfected HEK293Ts with CAP88 T/F and CAP88 12M IECs and visualising the virions that were bound following multiple washes by staining with p24-FITC (Figure 2.6A). Both CAP88 T/F and CAP88 12M IECs bound to $\alpha4\beta7^{+}$ HEK293T cells but not to α4β7- cells. Furthermore, the proportion of $p24^{+}$ HEK293T cells was significantly decreased by treatment with α4β7 specific mAbs Act-1 and HP2/1 for the CAP88 T/F virus as shown in Figure 2.6B. Concentrations used for Act-1 and HP2/1 were those

optimal for blocking HIV replication, as these are most relevant for this study. The proportion of $p24^+$ cells was significantly higher when incubated with CAP88 T/F and 12 month IEC was also significant shown in Figure 2.6C; this finding mirrors binding observed using gp120 monomers from these same viruses in a previous study (Nawaz, et al., 2011).

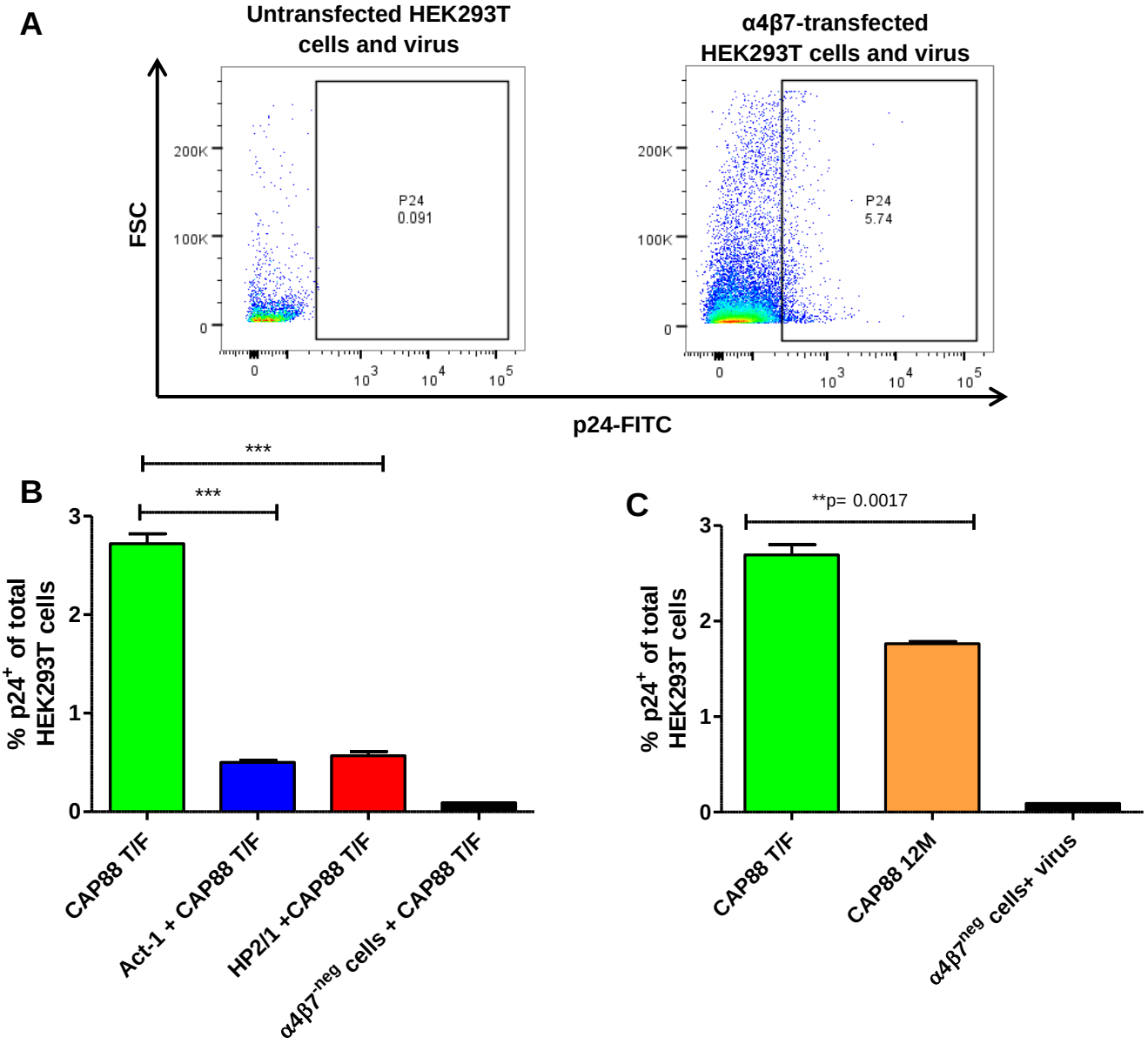


Figure 2.6: Binding of IECs to α4β7 in a direct virus binding assay
(A) $p24^+$ HEK293T cells were distinguished in the negative control (HEK293T cells untransfected with α4β7 and incubated with virus) and HEK293T cells transfected with α4β7 (left and right panels respectively), matched for the virus of interest. **(B)** This increase is abrogated significantly by inhibition controls Act-1 and HP2/1 (blue and red bars), indicated by a decrease in the proportion of $p24^+$ HEK293T cells (*Repeated measures one way ANOVA with Tukey multiple comparisons post test*). **(C)** The difference in proportion of $p24^+$ cells between the CAP88 T/F and its 12 month clone is significant (*p=0.0017;paired t test)*. These experiments consist of four replicates with bars indicating the mean of these and the error bar, the SEM.

The second binding assay was performed by incubating α4β7^{+} HEK293T cells with CAP88 T/F and 12M IECs followed by incubation with α4-PE and β7-FITC mAbs. Percentage binding to α4β7 was represented by percentage difference between the MFI of the experimental control (not incubated with virus or inhibitory mAbs) and α4β7^{+} HEK293T cells incubated with virus or inhibitory mAbs HP2/1 and Act-1 (Figure 2.7 B), Results recapitulated observations made in the direct binding assay: CAP88 T/F and CAP88 12 month IECs bound to α4β7, with significantly more binding of the CAP88 T/F IEC to α4β7 as compared to CAP88 12 month IEC for β7-PE. .Positive controls Act-1 and HP2/1 also bound to α4β7.

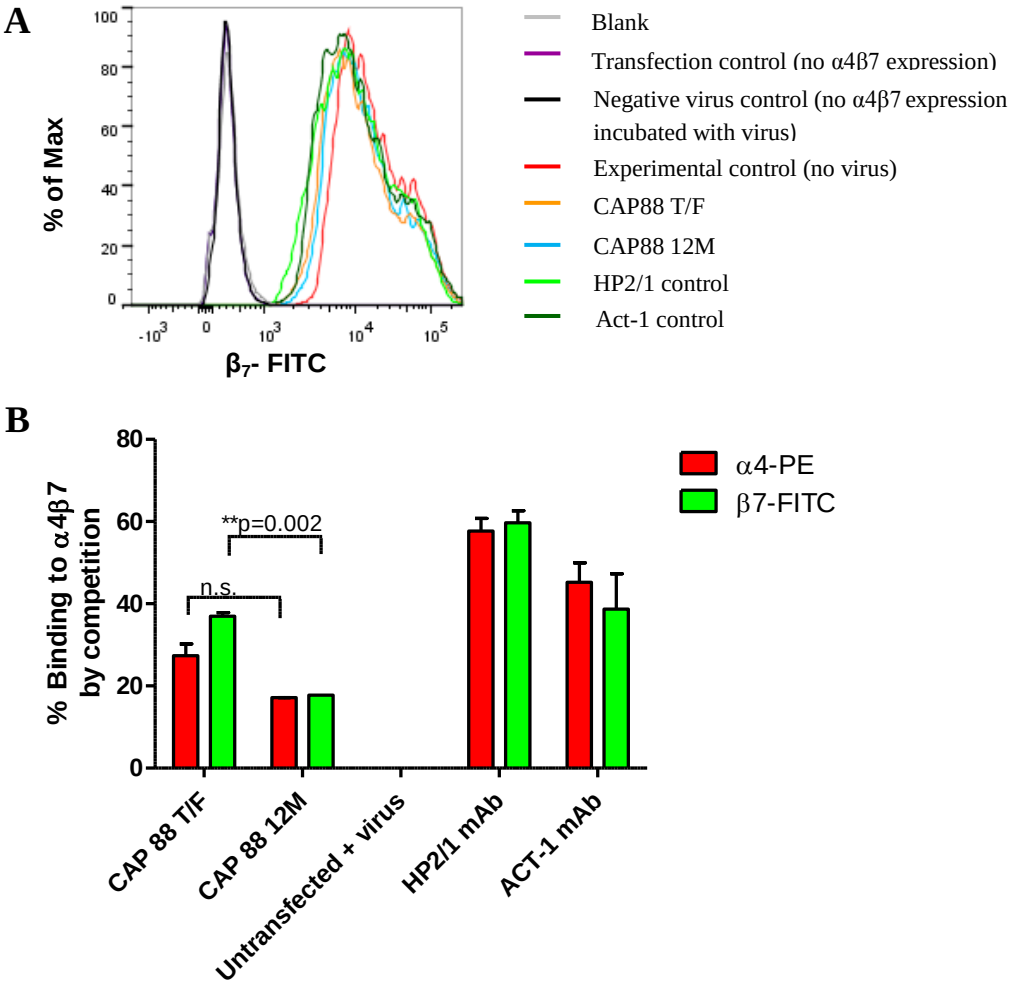


Figure 2.7: Binding of IECs to α4β7 in a competition flow assay
(A) HEK293T cells transfected with $\alpha_4\beta_7$ bound IECs representative of early (orange) and chronic (blue) infection as shown by the decrease in β_7–FITC fluorescence relative to the experimental control (transfected 293s with no bound virus, red). HP2/1 and Act-1 binding were used as positive controls (light and dark green respectively) while the negative control was α4β7 untransfected cells incubated with virus. All populations were gated on α4β7^{+} live single 293s. (B) The percentage difference between the MFI of both α4-PE and β7-FITC (red and green respectively) and the experimental control were used calculate an inferred binding capacity. Difference between CAP88 T/F and CAP88 12M binding was significant by competition with β7-FITC (p=0.002 for β7; Paired t test, n=3).

It has been suggested previously that as α4β7 is an attachment factor, it is likely that the integrin would play a greater role in facilitating viral replication when viral input is low (Parrish, et al., 2012). To this end, we sought to determine whether IECs CAP88 T/F and CAP88 12 months preferentially bound to α4β7 at lower viral inputs. This was not the case in either virus with all concentrations yielding similar levels of binding represented by α4-PE and β7-FITC within a range of 0.5 – 25 ng (Figure 2.8 A and B).

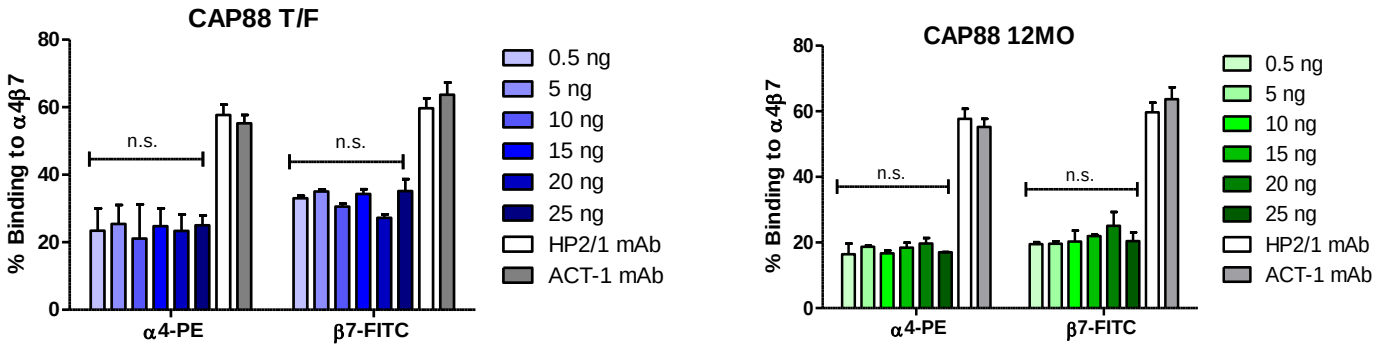


Figure 2.8: Binding of IECs to α4β7 is not altered by viral input
Percentage binding of **(A)** CAP88 T/F and **(B)** CAP88 12 month clone did not vary with amount of virus (ng) in an α4β7-dependent HEK293T cell assay when compared in terms of median fluorescence intensity of either α4-PE or β7-FITC (*Repeated measures ANOVA with Tukey multiple comparison post test*). These data are representative of two independent experiments and bars indicate means while error bars indicate SEM.

2.3.3. Influence of α4β7 on HIV replication changes longitudinally over time

Following the finding that IECs were able to bind α4β7 and appear to do so differentially, similar to a range gp120 monomers that have been tested (Nawaz, et al., 2011; Arthos, et al., 2008), we sought to determine whether α4β7 a) has a role in HIV replication and b) if so, whether the role changes over time.

An α4β7-inhibition assay involving the incubation of ATRA treated $CD4^+$ T cells with HP2/1 mAb (targets α4 subunit only; the point of contact for gp120) and Act-1 mAb (targets the α4β7) was performed. The cells were infected with viruses from 11 different individuals inclusive of time points from the point of transmission, 1 or 2 months post infection (p.i.) and chronic infection (6, 12 and 39 months p.i.) and viral kinetic growth was monitored by p24 ELISA over 10 days. As described in

Chapter 2 Section 2. 3.1, titration of the inhibiting mAb was crucial to accurately determine the effect of α4β7 on HIV replication. Viral replication in the presence of 275 pM HP2/1 and 2.2 pM Act-1 treated cells is similar at the point of exponential growth of the virus control. This is illustrated in Figure 2.9, using a T/F virus from participant CAP225. The use of HP2/1 and Act-1 mAbs head-to-head is a control that confirms the inhibitory effect on viral growth is as a result of the advantage that α4β7 offers to replication. In addition, the effect of these inhibitory mAbs on virus replication is only partial while blocking the CD4 receptor completely abrogates viral replication.

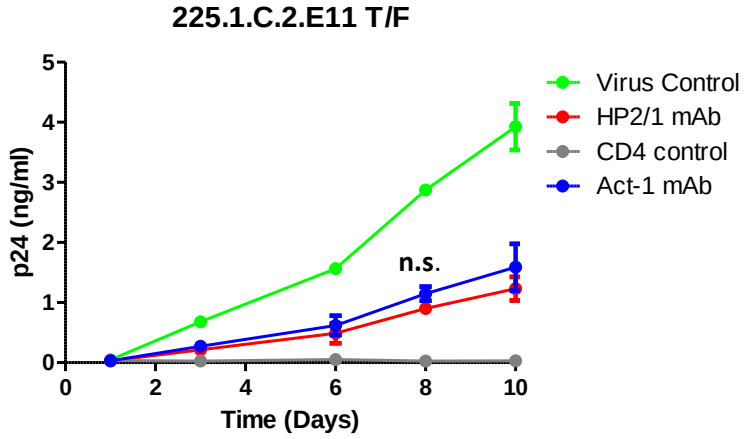


Figure 2.9: Act-1 mAb and HP2/1 mAb inhibit α4β7-dependent replication with similar efficacy
Virus kinetic growth curves of a T/F from CAP225 without (green) or with an α4β7 inhibitory mAb Act-1 (blue) and HP2/1 (red) and an infectivity control in the form of an anti-CD4 mAb (grey) measured as p24 (ng/µl) over 10 days. Inhibition by Act-1 and HP2/1 at the point of exponential growth of the virus control was not significantly different represented by n.s at day 8 (*Wilcoxon matched pairs signed rank test n = 3*). This is representative of three independent experiments with dots representing the mean of three replicates and error bars representative of SEM.

Comparison between the α4β7 reactivity of IECs and IMCs

It has been suggested that viruses generated using a standard HIV backbone, as in this study, do not allow the effect of gene products other than Env to the T/F phenotype to be assessed and that the potential for the overexpression of Env in this model compared to naturally occurring viruses prohibits a meaningful assessment of virus–host cell interactions (Parrish et al., 2013). As a result it was necessary to compare the dependence of our unique IEC virus construction to the dependence of matching IMCs on α4β7. As IMCs consist of a proviral genome completely representative of the virus from a particular individual and IECs are only representative of a particular virus in terms of the *env*

gene. It is possible that a variety of factors purely related to the chosen virus expression system may influence the dependence on α4β7 including opportunistic recombination and higher expression the env glycoprotein compared to IMCs. As shown in Figure 2.10 both binding to and dependence for replication on α4β7 were not significantly different between matched IECs and IMCs for two participants (CAP210 T/F and CAP239 T/F viruses). This suggests that the IEC system is comparable to that of IMCs and is a suitable model for viral replication in this study.

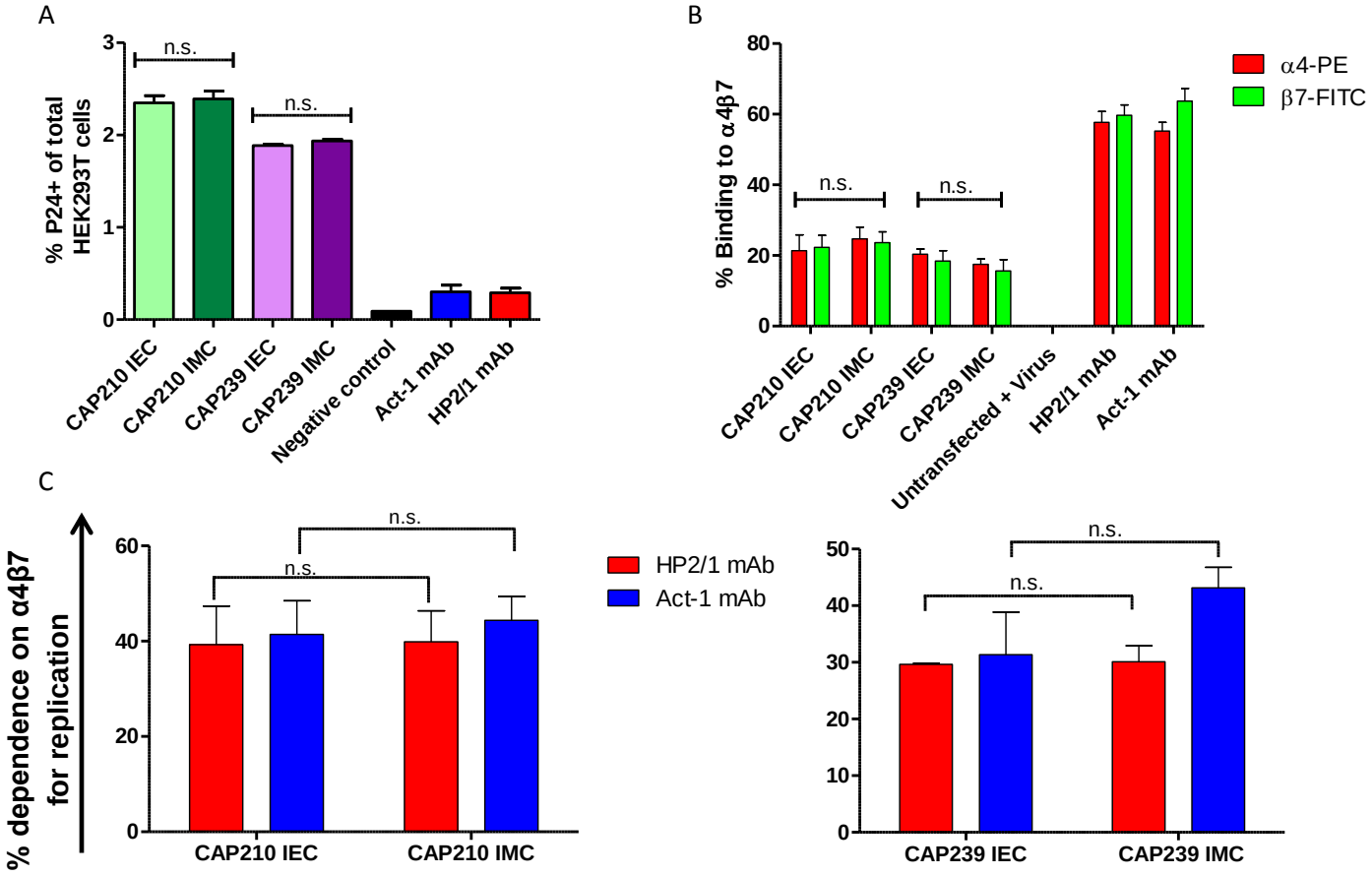


Figure 2.10: IMCs and IECs have similar binding and replicative dependencies on α4β7
(A) Direct p24 binding assay shows no significant difference between IEC and IMC binding to α4β7. **(B)** This is mirrored by the competition binding assay when competed with α4-PE (red) or β7-FITC (green). **(C)** Dependence on α4β7 for replication is not significantly different between IECs and IMCs (CAP210 and 239) in the case of both HP2/1 (red) and Act-1 (blue). All comparisons were non-significant by the paired t test where each experiment is representative of 3 independent experiments.

Longitudinal dependence of viruses on α4β7 for replication

Of the IECs available in this study, three individuals (CAP88, CAP200, CAP206) have a completely representative data set; namely a T/F virus, a clone within 1 year of infection (1/2 months or 6 months), a clone at 12 months and 39 months (those data sets in the black box in Figure 2.11). These

data show a similar pattern whereby the T/F virus has a higher dependence on α4β7 for replication which decreases after transmission and is maintained at these levels in the first year of infection and increases at 3 year p.i. As a result of the viruses in these three individuals being matched, this pattern is of particular relevance. Kinetic growth curves of virus clones of all individuals are shown in Table B2 of the Appendix and results are summarised in Figure 2.11.

In order to determine why the dependencies of T/F viruses from CAP88, CAP200 and CAP206 on α4β7 differ the most dramatically from their next available viral clones (2 months p.i. in the cases of CAP88 and CAP206 and 6 months in CAP200 indicated in Figure 2.11) I aligned their gp160 sequences (available from previous studies from our laboratory) to HBX2 (shown in Figure 2.12). This sequence is one that is used to standardise the numbering of sequence positions in an alignment. The alignment also shows the designation of the variable loops (V1-V5) and one constant region (C3) mentioned throughout this study. In addition, the position of N-linked glycans (indicated in red) was predicted by N-glycosite, a tool available on the Los Alamos National Database (Zhang, et al., 2004). While no common themes can be drawn from this analysis it is intriguing that the only changes in sequence in the case of the CAP88 T/F (88.2.00.17.-5A) and the 2 month clone, CAP88 2.00.B5 are 3 amino acid changes in gp41, one of which (Leu 568 Arg) is highly conserved in the N-heptad repeat of gp41. Not only has the conservation of this residue been associated with membrane fusion (Dwyer et al., 2008) but it is a mutation that disrupts the very hydrophobic nature of this region. Furthermore, it has been associated with a reduction in the ability of gp120 expressing cells to fuse and form syncytia with target cells that express the appropriate receptors (Bar and Alizon, 2004). As a result of $\alpha4\beta7^{+}$ T cells being able to homotypically aggregate (Zeller et al., 2001) and the ability of the ability of the integrin to activate LFA-1 virological synapse formation, the reduction of the virus' dependence on α4β7 appears to be as a result of the hydrophyllic nature of Arg 568 (present in CAP88.2.00.B5).

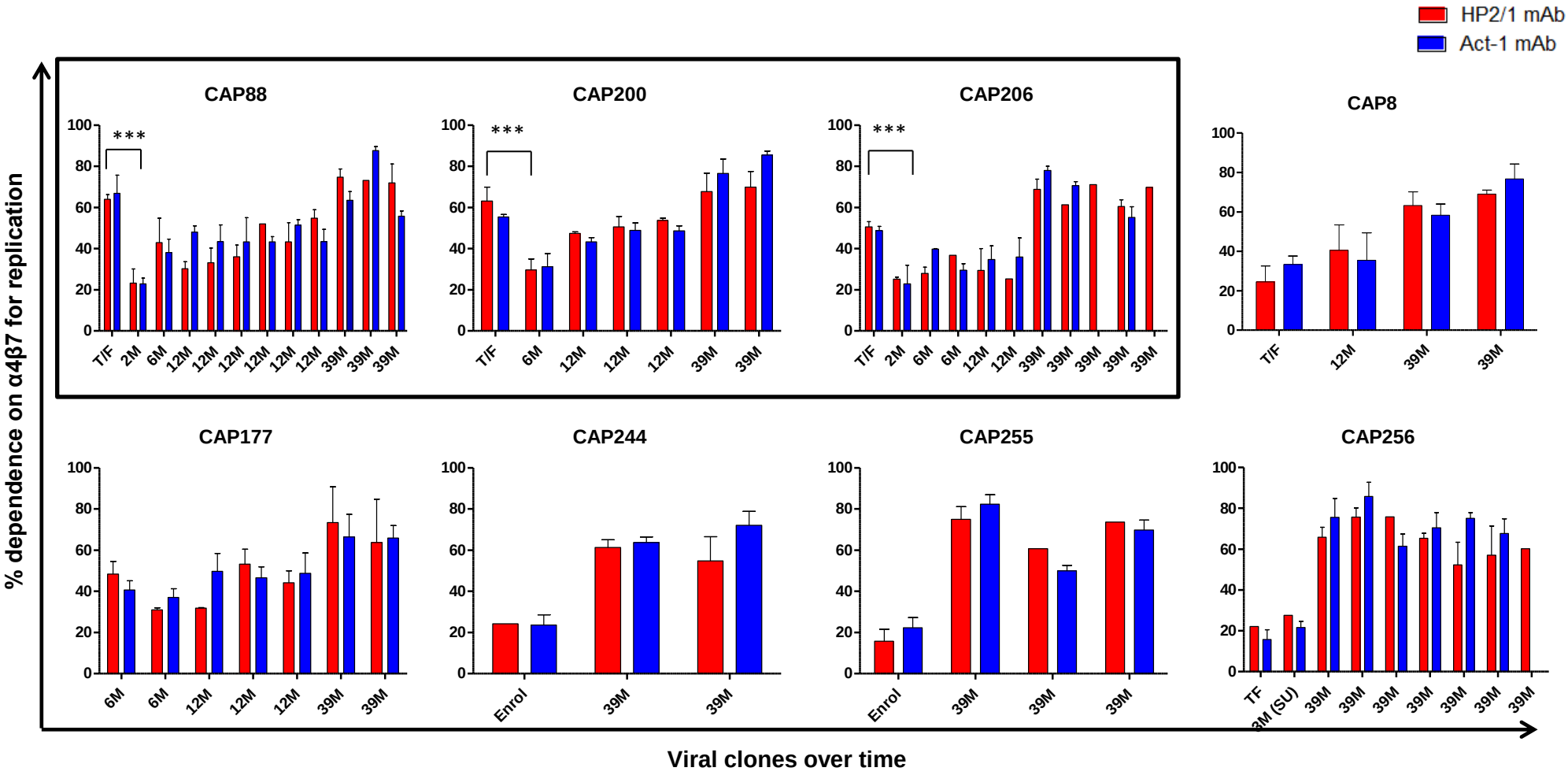


Figure 2.11: The role of α4β7 changes over time

Virus clones derived from eight individuals including T/F viruses and 1-39 months post-infection (p.i.) time points were tested for their dependence on α4β7 for viral replication calculated as percentage inhibition by HP2/1 (red) and Act-1 (blue) expressed as percentage difference of p24 concentration between HP2/1 or Act-1 treated and untreated virus in exponential growth phase. The box denotes individuals CAP88, CAP200 and CAP206 with a complete data set namely T/F, 2 months (absent in CAP200), 6, 12 and 39 months p.i. which follow a distinct pattern of α4β7 usage over time. Differences in dependence across all time points in each of these three individuals were significant by a repeated measures ANOVA for both Act-1 and HP2/1(*** $p<0.0001$) as well as between CAP88 T/F and 2 months p.i, (***$p<0.0001$ for HP2/1 and **$p<0.001$ for Act-1) CAP200 T/F and 6 months p.i. (***$p<0.0001$ for HP2/1 and *$p<0.01$ for Act-1) and CAP206 T/F and 2 months p.i (***$p<0.0001$ for HP2/1 and **$p<0.001$ for Act-1), Pairwise comparisons were adjusted by the Tukey method. Bars represent means of between two and three independent experiments with the error bars indicating SEM.

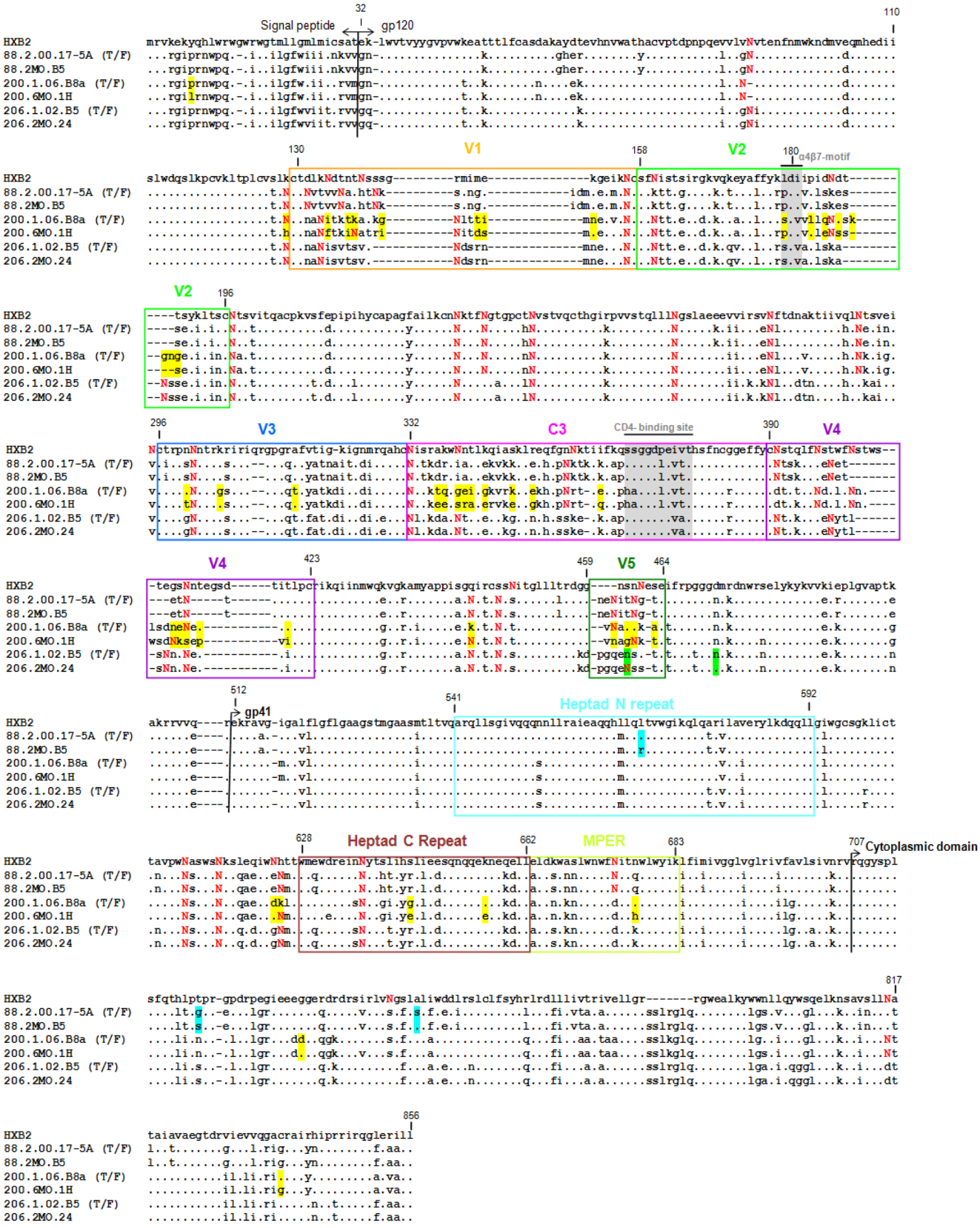


Figure 2.12: Sequence alignment of CAP88, CAP200 and CAP206 gp160s
The T/F and 2 or 6 month p.i. gp160 sequences of CAP88, CAP200 and CAP206 aligned to HBX2. Glycans are indicated in uppercase and highlighted in red. The α4β7 binding motif and CD4 binding sites are highlighted in grey and the non-synonymous sequence changes between CAP88 T/F and 2 month, CAP200 T/F and 6 month and CAP206 T/F and 2 month highlighted in blue yellow and green respectively. Different structural regions of gp120 are denoted by coloured blocks and dots represent identities.

Three amino acid differences exist between CAP206 T/F and the 2 month clone, one of which is a glycan at position N461 in the 2 month clone. Situated in V5, a region linked to CD4-binding this forms part of the conformational epitope for the CD4-binding site mAb VRC01 (Li et al., 2011b). This change may result in a reduced affinity of the virus for α4β7 because CD4 is close enough to α4β7 on the cell surface for them to be co-precipitated (Cicala, et al., 2009). While differences in sequence between CAP200 T/F the 6 month clone are far more extensive (39 non-synonymous changes, consistent with the longer duration of infection), it is likely that the change of the α4β7-binding motif from SDV to PDI at 6 months is significant and CAP200 is the only individual that has a non-synonymous motif change over time. All motif sequences are shown in the Appendix (Table B2).

Although these changes are intriguing, many are unique to individuals and as a result it was necessary to determine factors inclusive of all individuals that may contribute to high α4β7 dependence for viral replication. The remaining five individuals depicted in Figure 2.11, which had matched T/F (CAP8 and CAP256) or enrolment clones (CAP244 and CAP255) and chronic clones, with the exception of CAP177 which due to availability had no very early clone were used to assess overall patterns of α4β7-dependence as shown in Figure 2.13. Whether Act-1 (Figure 2.13 A) or HP2/1 (Figure 2.13 B) is used as the defining parameter, T/F viruses have a significantly higher dependence on α4β7 for replication and significantly lower than that of 39 month viruses. Strikingly, there is no significant difference in α4β7 dependence between T/F viruses and chronic clones at 6 and 12 months. Overall, this mirrors the finding that there is no difference between T/F viruses and chronic viruses in the first year of infection (Parrish, et al., 2012). Despite this, the pattern of dependence still suggests that α4β7 may play an important role in transmission which diminishes shortly after infection. These data suggest that over time, the dependence on α4β7 for viral replication changes.

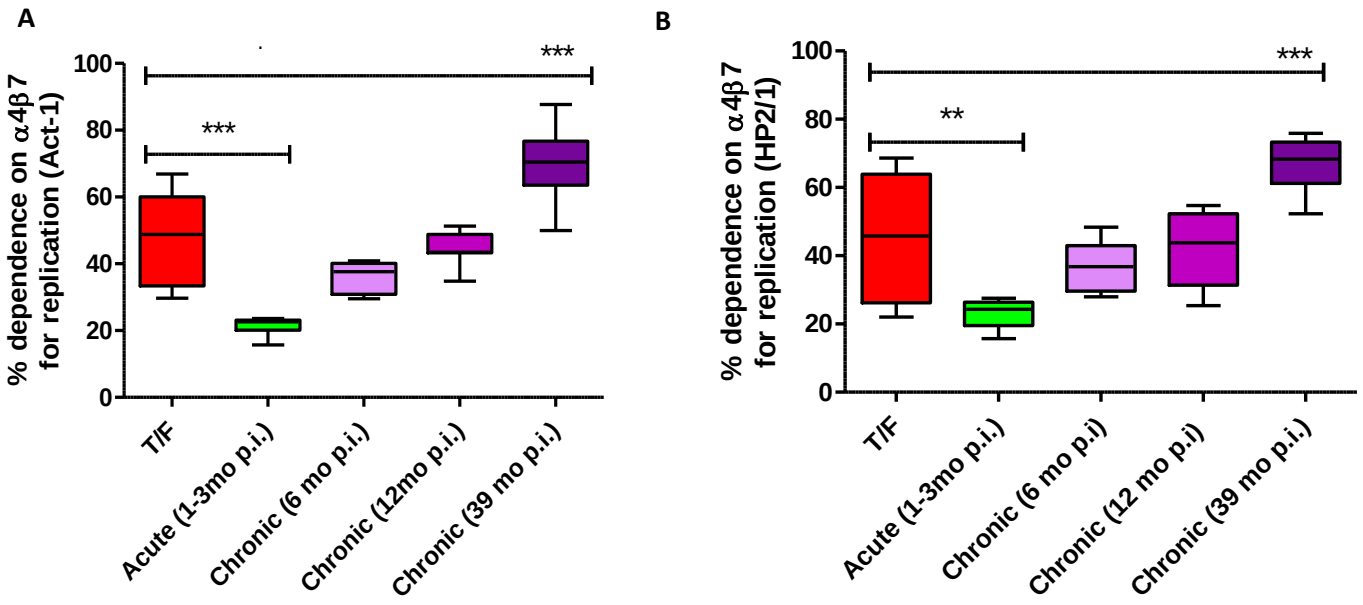


Figure 2.13: Pattern of α4β7 usage changes during different clinical stages of HIV
T/F viruses (n=8) had a significantly higher dependence on α4β7 for replication than acute viruses (n=5) (1-3 months p.i.) as determined by both **(A)** Act-1 (***p<0.0001) and **(B)** HP2/1 mAb (***p<0.001*), no significant difference compared to viruses from the first year including both 6 (*n=6*) and 12 months p.i. of chronic infection (*n = 15*) and a significant decrease compared to viruses 3 years p.i. (n=24, *p<0.0001 for both Act-1 and HP2/1)*. Both data sets were analysed for significance by a one way ANOVA and pairwise comparisons adjusted for by Tukey's method.

2.3.4. Factors associated with high α4β7 dependence

In order to determine why certain viruses have a higher dependence on α4β7 for viral replication, a number of additional viral factors were investigated. The reduced length and glycan density of the V1/V2 loop as well as C3/V4 has been associated with increased α4β7 binding (Nawaz, et al., 2011) although, a recent study by Etemad, et al., 2013 suggests that in matched samples this is not the case at least for the V1/V2 loop. We sought to determine what the association of length and glycan density in all variable loops as well as C3/V4 was with dependence on α4β7 for viral replication as seen in Figure 2.14 A and B. Sequences from previous studies in our laboratory corresponding to the env proteins of the viruses used in this study were analysed for number of glycans as predicted by the N-glycosite tool from the Los Alamos National Database (Zhang et al., 2004a). Several linear Spearman correlations were significant shown in grey blocks in Figure 2.14 including the glycan density of V2, V1/V2, V4 and C3/V4 and the length in amino acids of V1/V2 and C3/V4. However because of the interrelatedness of these regions, it was necessary to use a multiple regression to assess which of these associations are significant when others are kept constant. To limit multicolinearity, redundant variables were excluded from the data set (namely V1, V2 and V4 with only V1/V2, C3/V4 and V5

regions included) and glycan density and variable loop lengths were analysed separately because of their causal relationship. Overall V1/V2 length and glycan density were positively associated (β-coefficient = 1.23 and 3.81 respectively) with the dependence on α4β7 for viral replication for Act-1 inhibitory mAb. A complete data set of all lengths and glycan densities are shown in the Appendix (Table B2) but the sequences have not be included. In this data set, the region on which the α4β7 binding site is present was most significantly associated with dependence on the integrin; similar to what has been hypothesised previously (Nawaz, et al., 2011).

Owing to glycan density and length of the V1/V2 region having an association with the effect of α4β7 on replication, we sought to determine whether the linear distance of the glycans from the α4β7 binding motif in V1/V2 has an impact on the efficacy of the integrin's effect on viral replication. To this end each individual viral clone was designated as exhibiting either high (green) or low (blue) α4β7 dependence for viral replication. This was done by comparing percentage dependence of each individual clone to the mean dependence on α4β7 (51.02 % with reference to HP2/1 mAb and 51.92% with reference to Act-1 mAb) with those above the mean designated as "high" and those below, "low". A full list of designations can be noted in the Appendix (Table B2) with 27 sequences determined as having low α4β7 dependence and 33 designated as having high α4β7 dependence.

The frequency of sequences (as a proportion of those viruses with a high or low α4β7 dependence respectively) expressing a glycan at a particular linear distance from the tripeptide minimal binding epitope for α4β7 in the V1/V2 loop is shown in Figure 2.15. This reflects the great variability of this region of gp120, in that only a small number of sequences (reflected by small percentages) have glycans at different linear distances from the motif which very few displaying common positions across a number of individuals. Glycans present 5 amino acids downstream and those 41 amino acids upstream from the motif are more frequent in viruses that have high affinity for α4β7 (8 versus 2 and 9 versus 3 sequences respectively) while glycans 8 amino acid downstream are more frequent in viruses with low α4β7 affinity (7 versus 2 sequences) with the numbers of sequences indicated above the respective bars.

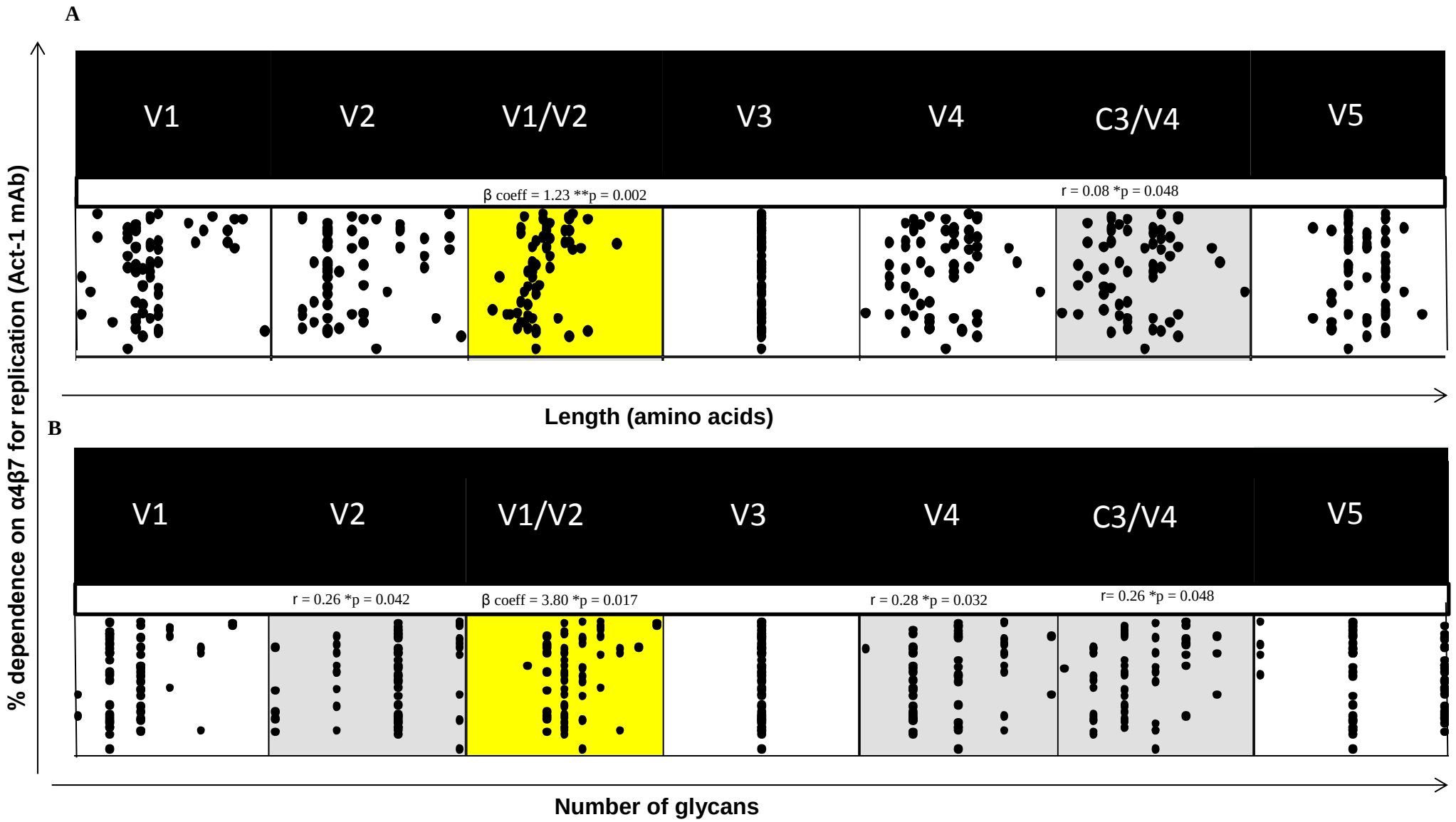


Figure 2.14: Association of length and glycan density of variable loops with dependence on α4β7 for replication
Correlations between the (A) length and (B) glycan density of V1, V2, V1/V2, V3, V4, C3/V4, V5 and dependence on α4β7 for replication with reference to both HP2/1 and Act-1 inhibitory mAbs. Blocks shaded in grey are correlations that showed a significantly positive correlation (Spearman's correlation) between the respective variables and yellow blocks are those associations that were significant by multiple regression. Beta coefficients, Spearmans coefficients and p values are shown above the respective blocks.

These differences are not significant using the Fisher exact test, nor is there a definite trend for positions closer to the motif to affect α4β7 reactivity either positively or negatively. While these numbers are small, the isolated cases where there are differences between viruses with high and low α4β7 dependence for replication suggest that glycans in specific positions within the gp120 protein might be of importance to α4β7 reactivity and perhaps in terms of their position in the trimer rather than their linear distance from the α4β7 binding motif.

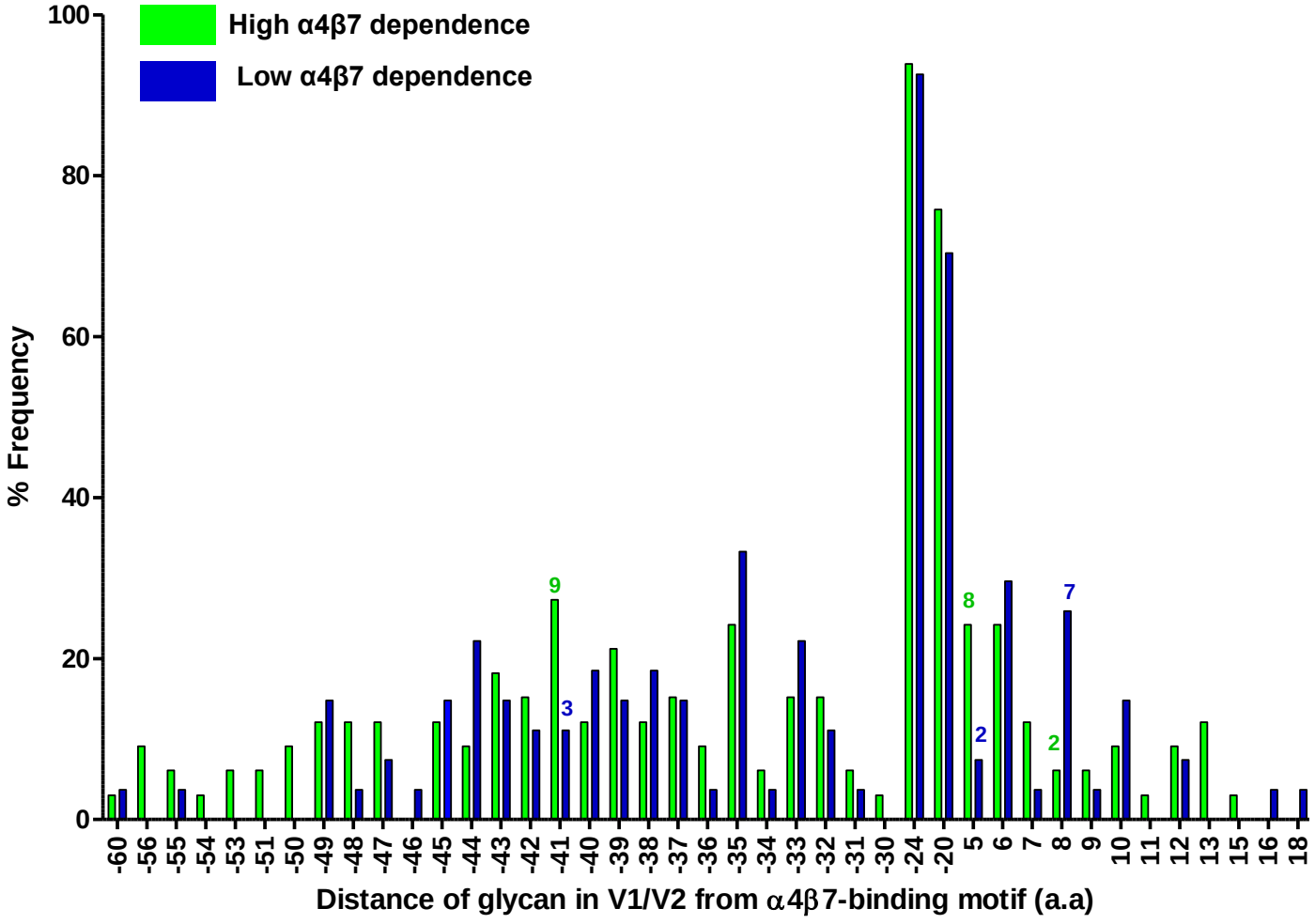


Figure 2.15: Distances of glycans from the α4β7 binding site in the V1/V2 loop of gp120
The frequency of distances of different glycans across 60 viruses from 11 individuals from the α4β7 binding motif is represented as a proportion of those viruses with high and low α4β7 dependence for viral replication (shown as green and blue bars respectively). Numbers above the bars indicate the quantity sequences represented for each case.

While the observations made in terms of density of glycans are of importance, it is possible that the presence or absence of particular glycans within gp160 may influence α4β7 dependence for replication and that these appear largely unrelated to linear distance from the α4β7 binding motif. Using a similar stratification of α4β7 dependence for viral replication, the frequency of sequences that have glycans at positions in gp160 as aligned to HBX2 were expressed as a proportion of either high or low α4β7 dependent sequences as shown in Figure 2.16. While the whole of the gp160 sequence was investigated (Figure B3Appendix), only those that appeared different between the two groups across at least three individuals were deemed as relevant. These 12 glycan positions are include 130, 150 (V1), 185 (V2), 234 (C2), 332, 334,339, 355, 362 (C3), 409 (V4), 448 (C4) and 462 (V5) shown in Table 2.2 while the location of these positions in the trimer is shown in Figure 2.17.

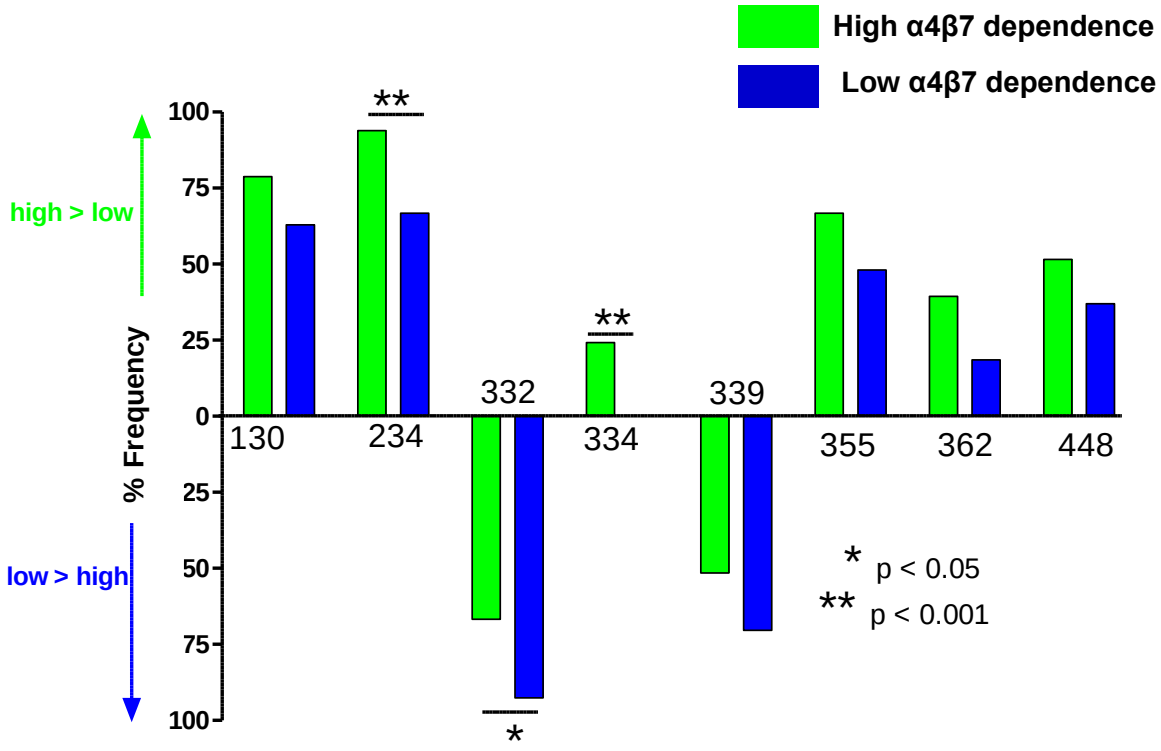


Figure 2.16: Frequency of glycans in conserved regions of gp120 showing differences between high and low α4β7 dependence

Frequency of glycans relative to the number of high or low α4β7 dependent viruses are shown in green and blue respectively, with those positions that show difference where high α4β7 dependent viruses have a higher frequency than low above the x axis and vis versa, below the axis. Significance was determined by the Fisher exact t test.

Table 2.2: Glycans associated with α4β7 dependence for viral replication
Glycan numbers highlighted in green indicate those which are more prevalent in viruses with a high dependence on α4β7 for replication while those in blue are more prevalent in viruses with a low dependence. The number of sequences with the respective glycan across individuals is shown in bold and the frequency as a percentage of high and low dependent viruses indicated in italics. The number of individuals in which the glycan appears is also represented.

Glycan	Region of gp120	Number of sequences		Number of individuals represented by sequences
		High α4β7 dependence	Low α4β7 dependence	
130	V1	**26** (*79*)	**17** (*63*)	11
150	V1	**12** (*36*)	**6** (*22*)	7
185	V2	**8** (*24*)	**2** (*7*)	4
234	C2	**31** (*94*)	**18** (*67*)	10
332	C3	**22** (*67*)	**25** (*93*)	11
334	C3	**8** (*24*)	**0** (*0*)	3
339	C3	**17** (*52*)	**19** (*70*)	9
355	C3	**22** (*67*)	**13** (*48*)	8
362	C3	**13** (*39*)	**5** (*19*)	4
409	V4	**14** (*42*)	**4** (*15*)	5
448	C4	**17** (*52*)	**10** (*37*)	8
462	V5	**15** (*45*)	**19** (*70*)	7

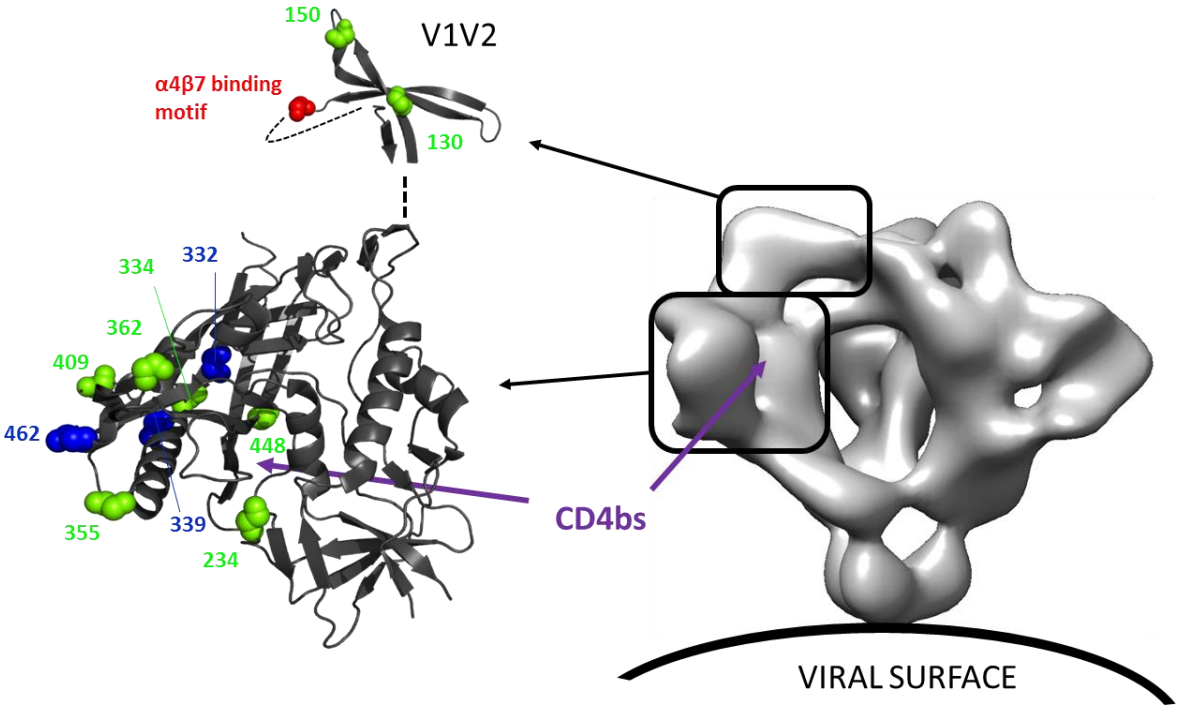


Figure 2.17: Position of glycans that impact on α4β7 reactivity in the gp120 trimer
Here the most recently available trimer structure (Mao, et al., 2013) was used to model the positions of those glycans that are more frequently present in viruses with high α4β7 dependence (green) and those with low dependence (blue). The α4β7 binding motif is indicated in red and the position of the CD4 binding site indicated in purple. Positions of 130, 185 and 409 are not necessarily accurate as they are in the variable loops which are different lengths in different viruses. The V1/V2 structure is modelled on that proposed by McLellan, et al., 2011). The model was created using the PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC.

Of the 12 notable glycan positions, only three showed significant differences between the two groups in the conserved regions of gp120 including N234, N332 and N334 (Figure 2.16). In addition, position N409 was also found to be significant and other differences were also noted in the variable regions of gp120 (N150, N185 and N462). However the importance of these in α4β7 reactivity is less clear because of the extreme range of variable loop length and it is difficult to accurately determine the position of the glycan in gp120. Only two of the glycans in the conserved regions have a higher frequency in viruses with a low dependence on α4β7 namely N332 and N339 (and N462 in the variable loops) while the remaining 9 are present in a higher frequency in viruses with a high dependence on α4β7 (N130, N185, N234, N334, N355, N362, N409, N448). Only three of the latter are present in the V1/V2 loop (N150 and N185 with N130 present in the V1/V2 β sheet) and are situated near the α4β7 binding site. Several of these are in close proximity to CD4 contact sites including N130, N362 and N355 (Zhou et al., 2010) which may allow for conformational stabilisation of the CD4-gp120 and α4β7-gp120 interactions as a result of the close proximity of CD4 and α4β7 on the surface of the target cell; so close in fact that these receptors were able to be co-precipitated (Cicala, et al., 2009).

N462 is a target for VRC01, a CD4 binding mAb, and is also located right next to a CD4 contact site in V5. However unlike the other glycans, this was found to be more frequent in viruses with low α4β7 dependence. As such, it is possible that the presence of a glycan at this position may sterically impede the affinity of the virus for α4β7. N234 is a high mannose glycan, associated with DC-SIGN binding (Alexandre et al., 2012) and its removal has been found to have a significant reducing effect on infectivity and Env incorporation (Wang et al., 2013), possible reasons for its selection in viruses with a high affinity for α4β7. This glycan is also in the region where gp120 and gp41 are thought to interact (Yang et al., 2003). N448 is a conserved high mannose glycan (similarly to N234). These glycans are required for protein folding and stabilisation of protein conformation (Wyss et al., 1995) and have been found to be more extensively represented and conserved in T/F viruses (Go et al., 2011) suggesting a link between these glycans and α4β7 in transmission.

The observation that N332 is more frequent in viruses with low α4β7 dependence is intriguing as it was found to be underrepresented in T/F viruses (Moore et al., 2012) and N332 knockouts have been found in our laboratory to have higher viral fitness than those with the glycan (Moore, unpublished). This suggests that this glycan may mitigate the selective advantage that α4β7 offers during replication. In addition, N334 is exclusive to high α4β7 dependent viruses which suggests that the shift in glycan not only confers resistance to PGT128 (Moore, et al., 2012) but may enhance α4β7 affinity. Glycans 339 and 332 are clustered together on gp120 (Scanlan et al., 2002) and may be the reason for N339 being expressed predominantly on those viruses with low α4β7 dependence. N339 is positioned in the α2 helix of C3. Overall these data suggest that glycans throughout gp120 and not just within V1/V2 are important in determining how dependent viruses are on α4β7 for replication.

Of course it is possible that many other factors determine α4β7 reactivity and its influence on viral replication. Dependence on α4β7 for replication is significantly different between the various minimal tripeptide α4β7 binding motifs found across individuals in the first year of infection (Figure 2.18). Viruses expressing PDI/L or SDI/V motifs are significantly more dependent on α4β7 for replication than those expressing LDI/T/L. The difference is not significant between these three groups in the third year of infection (not shown). The tripeptide motif is highly conserved within an individual and as such differences do not explain changes in dependence longitudinally in individuals. Despite this, it is the first report of the sequence of the α4β7 motif affecting the dependence on the integrin for replication.

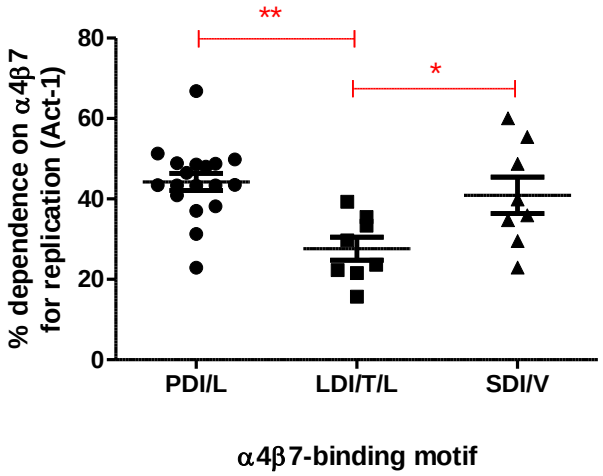


Figure 2.18: Different α4β7 tripeptide motifs affect dependence on α4β7 for replication in the first year of infection

Tripeptide loops PDI/L (n = 18) and SDI/V (n = 8) are expressed on viruses with higher dependence on α4β7 for viral replication than those expressing LDI/T/L (n = 8) (** *p<0.001 and *p< 0.01 respectively; one way ANOVA, pairwise adjustments by Tukey method*). Error bars represent the SEM and the lines represent the means.

It was possible to correlate the dependence on α4β7 for replication with disease outcomes including CD4 count and viral load (Figure 2.19A and B respectively) matched for each time point. Raw data are in Table B2 in the Appendix. No significant correlation with viral load (represented as log RNA copies/ml) was evident however a significant negative correlation was shown between α4β7 dependence and CD4 count (represented as cells/μl). This suggests that as the disease progresses and CD4 counts decline, dependence on α4β7 for replication increases correlating in part with the observation that 3 years post infection, individuals had the highest dependence on α4β7. This correlation may also reflect that *in vivo* at time points post infection where viruses that have high α4β7 dependence are dominant systemically, CD4 T cells may increase in their capacity to home to the GALT diminishing their presence in the blood. CD4 counts undertaken in the gut may reveal a positive correlation with α4β7 dependence of systemic viruses. These data were not available for analysis. This model does not include all T/F viruses as at the correlating time points, CD4 count was not performed.

As shown in Table B1 in the appendix, the individuals in this study have been stratified into rapid and intermediate progressors as well as those individuals that go on to develop broadly-cross neutralising (BCN) antibodies. No significant difference in dependence on α4β7 between rapid and intermediate progressors was noted at any time point examined (data not shown).

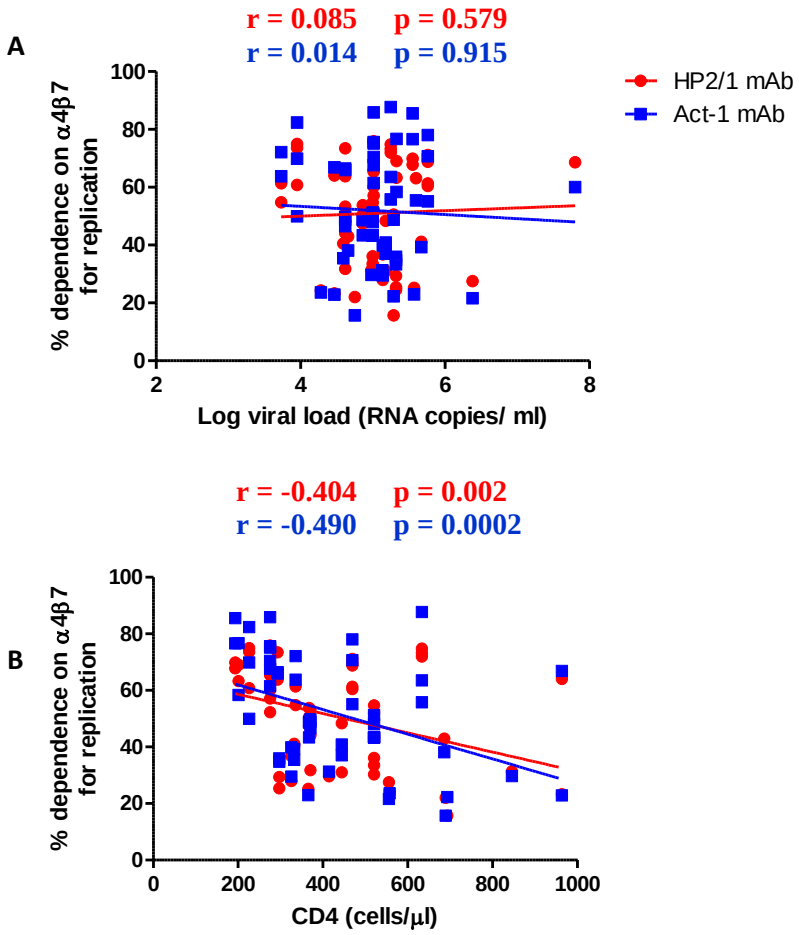


Figure 2.19: Correlation of HIV disease outcomes with dependence on α4β7 for replication
(A) There is no significant correlation between α4β7 dependence and log viral load (RNA copies/ml) for either HP2/1(red, n= 60) or Act-1 (blue n=57) while there is a significant negative correlation **(B)** between α4β7 dependence and CD4 count (cells/µl) for both Act-1 and HP2/1.

However, T/F viruses from individuals that go on to develop BCN antibodies showed a significantly lower dependence on α4β7 for replication than those that do not (Figure 2.20). Differences within the other time points were not significant. While this is not conclusive and requires further investigation, it is possible that the transmission event in individuals that go on to produce BCN antibodies is different to those that do not. These data suggest that BCN individuals do not make use of α4β7 to as great an extent as non-BCN individuals. As viral load has been associated with neutralisation breadth in BCN individuals (Doria-Rose et al., 2010; Gray et al., 2011) and α4β7 dependence does not correlate with viral load it is possible that α4β7 reactivity may be an additional factor responsible for initial differences in immunity. What does cause the difference is not known as very little is known about differences between BCN and nBCN individuals. This would need to be explored further but does suggest that BCN individuals have fundamentally different pressures at transmission that result

in viruses that have a decreased dependence on α4β7 for replication. It may be important to discern these pressures so we can improve the elicitation of effective BCN antibodiesin individuals.

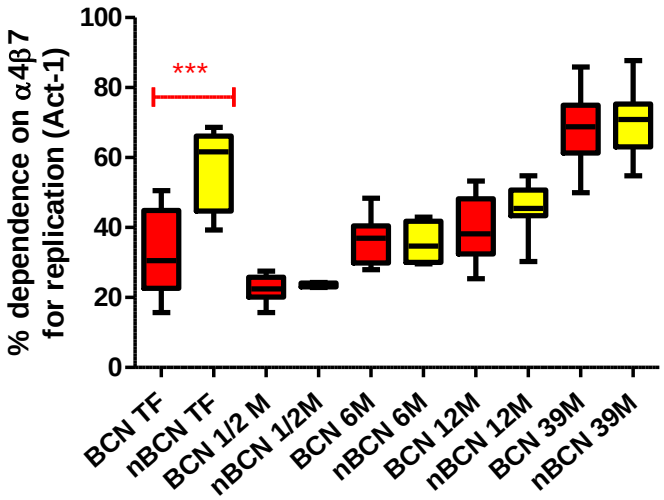


Figure 2.20: T/Fs of non-broadly cross neutralising (nBCN) individuals display higher dependence on α4β7 for replication than BCN individuals
Individuals that produce broadly cross neutralising antibodies two years post infection display significantly lower dependence than nBCN individuals on α4β7 for replication but only at the T/F stage (BCN T/F n=4; nBCN n=4; ***p<0.0001, one way ANOVA, pairwise corrections by Tukey method). Dependence on α4β7 is noted here based on Act-1 and HP2/1 inhibition.

2.3.5. The effect of STI status and cytokine milieu in the genital tract on the role of α4β7 in transmission

When the dependence on α4β7 for replication is compared across all the T/F viruses the range is broad as shown in Figure 2.21 (22-68.8% for HP2/1 and 15.7-66.9% for Act-1). While overall T/F viruses displayed high α4β7 dependencies, we sought to investigate if the wide range could be explained by factors that would be relevant at the point of transmission.

Following sequence analysis, the tripeptide α4β7 binding motifs present in the V1/V2 loop (shown in Figure 2.22A) could be used to stratify viruses with higher α4β7 dependence for replication (green) as compared to those with lower dependence (blue) (Figure 2.22 B). Those with high affinity had P/SDI/V binding motifs while those with low affinity had LDI/L motifs. While these differences may explain the variation in α4β7 dependence, it is important to consider the context in which transmission occurs.

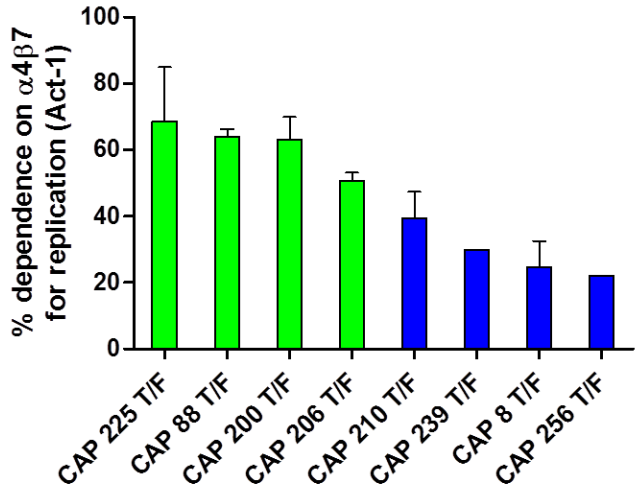


Figure 2.21: T/F viruses have a wide range of α4β7 dependencies for viral replication
T/F viruses exhibited a wide range of dependencies on α4β7for replication with CAP88, 200, 206 and 225 showing high α4β7 dependence (green) and the remaining four showing low α4β7 dependence (blue). Dependence was determined by Act-1 while bars represent the mean of four independent experiments, with error bars indicating SEM.

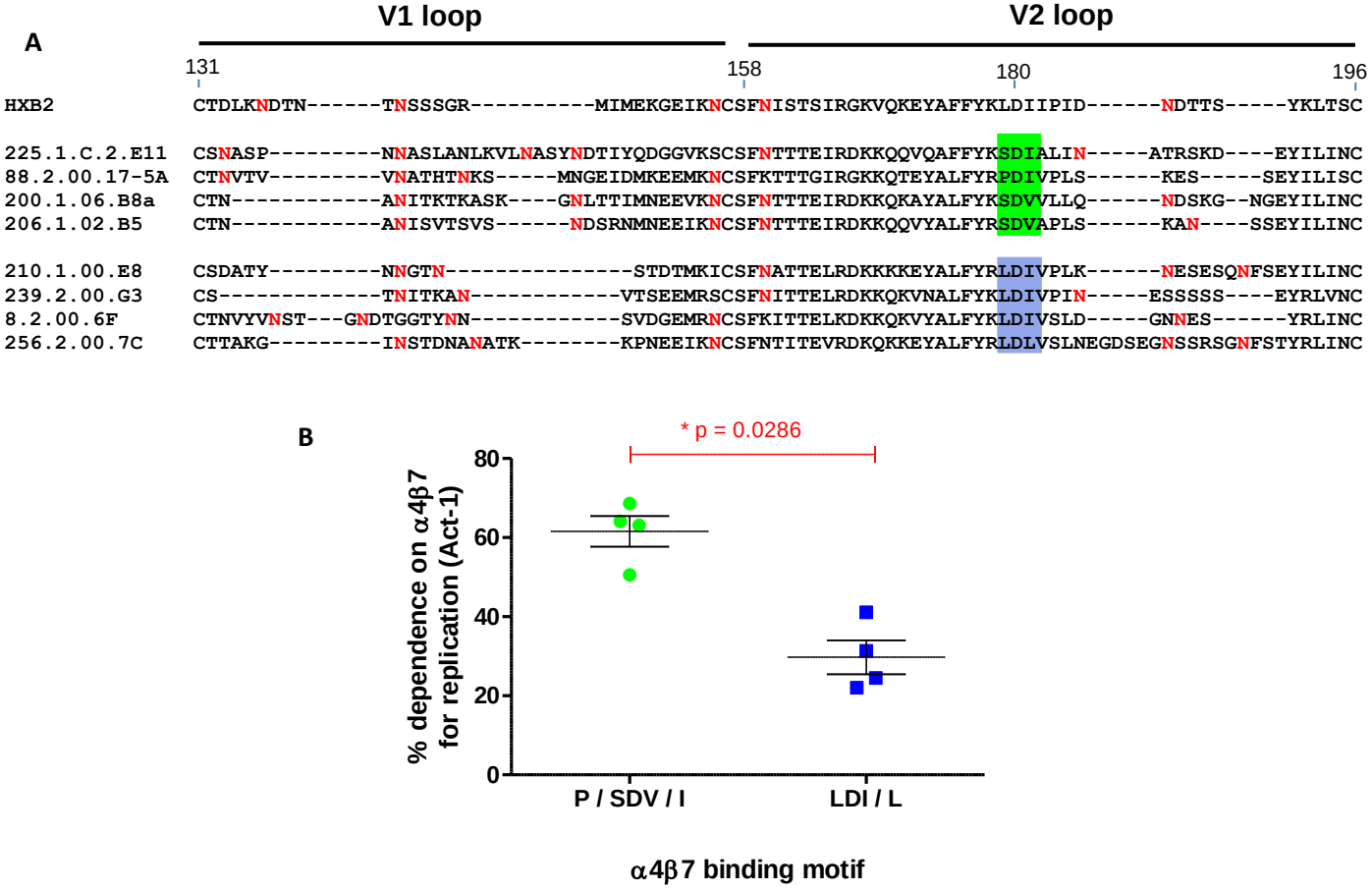


Figure 2.22: α4β7 dependence variation of T/F viruses may be attributed in part to α4β7-binding motif differences in the V1/V2 loop of gp120
(A) Sequence alignment of the V1/V2 loop of gp120 from the T/F with a highlighted α4β7-binding motif based on high (orange, n=4) or low dependence (green, n=6) on the integrin for replication graphically represented in B. LDI/L T/Fs have lower dependence on the integrin compared to those with P/SDI/V motifs (p=0.0286, Mann-Whitney test)

STIs have been identified as a major cause of inflammatory cytokine upregulation and immune cell recruitment to the genital mucosa (Levine et al., 1998). In addition to STIs and associated inflammation, bacterial vaginosis (BV) a syndrome characterised by a shift in vaginal flora composition (Fredricks et al., 2005), has been determined as a risk factor for HIV acquisition (Cohen et al., 2012; Nwadioha et al., 2011) as well as in a univariant analysis using the CAPRISA cohort but was not significant in a multivariant model (Mlisana et al., 2012). The homing function of α4β7, whereby cell subsets are able to traffic to the GALT and other extra-lymphoid tissues such as the genital mucosa, allows for cells predominantly of effector function to infiltrate inflamed tissue. Given this function and the integrin's demonstrated role in supporting viral replication of HIV, it is possible that the STI status of the individual at the time of transmission as well as the cytokine milieu present at the site of sexual transmission may influence whether α4β7 has a large or diminished role in the establishment of infection. To this end we obtained clinical data from the CAPRISA 002 cohort on the presence of various STIs (*Trichomonas vaginalis, Chlamydia trachomatis*, HSV-2, *Treponema pallidum*, *Neisseria gonorrhoea* and *Mycoplasma genitalium*) as well as BV at the time of transmission in the 8 individuals with T/F viruses (Table 2.3). While no HSV-2, Treponema pallidum, Neisseria gonorrhoea and Mycoplasma genitalium was detected in these individuals, *T. vaginalis, C. trachomatis* and BV were present. Strikingly those individuals that tested positive for BV at the time of HIV transmission had a significantly higher dependence α4β7 than those that did not (Figure 2.23). These data suggests that BV creates a transmission bottleneck whereby viruses that have a high dependence on α4β7 for replication are selected although the numbers are small.

Table 2.3: STI status of individuals at the point of HIV transmission[†]
Green shading indicates a group of T/F viruses that have dependence on α4β7 for replication below 50%, while green shows those with high dependence. BV= Bacterial vaginosis, *T. vaginalis* = Trichomonas vaginalis, *C. trachomatis* = Chlamydia trachomatis.

[†]HSV-2, *Treponema pallidum* (Syphilis), *Neisseria gonorrhoea*, *Mycoplasma genitalium* were negative for all samples and therefore not included in the table

		BV	*T. vaginalis*	*C. trachomatis*	α4β7-dep (Act-1)	α4β7-dep (HP2/1)
CAP 225	T/F	x			60.08	68.61
CAP 88	T/F	x	x		66.85	64.08
CAP 200	T/F	x			55.43	63.12
CAP 206	T/F	x			48.76	50.55
CAP 210	T/F				39.28	47.10
CAP 239	T/F				29.73	31.34
CAP 8	T/F			x	33.34	24.50
CAP256	T/F				15.72	22.00

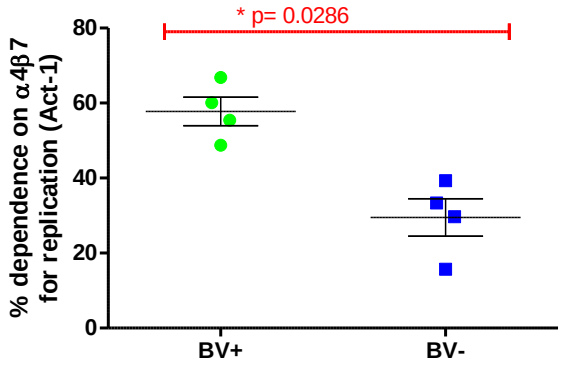


Figure 2.23: Bacterial vaginosis (BV) at the time of HIV transmission is associated with a high dependence of T/F viruses on α4β7 for HIV replication
Individuals that were BV positive (n=4) at the time of infection have a significantly higher dependence on α4β7 than those that were BV negative (n = 4) (p = 0.0286; Mann-Whitney test).

While it is not clear how BV may impact on the properties of viruses that establish infection, apart from an increase in the number of cells expressing α4β7 attracted to the genital mucosa in response to inflammation, a possible explanation is the cytokine milieu in a particular individual at the point of transmission that is present in the genital tract drives either expression of α4β7 or its ligand MAdCAM-1. We investigated the association between 31 inflammatory cytokines present in the cervicovaginal lavage (CVL) at the time of transmission of seven individuals with the matching T/F virus' dependence on α4β7 for replication. The cytokine levels of the corresponding individuals were

measured by luminex and the data obtained for another study (Masson, in review). The panel of cytokines tested included pro-inflammatory cytokines (IL-1α, IL-1β, IL-6, IL-12P40, IL-12P70, TNF-α); chemokines (Eoxtaxin, Fractalkine, IL-8, IP-10, MCP-1, MIP-1 α, MIP-1β, MIP-3A, RANTES); innate cytokines (IFN-α); haematopoetic (G-CSF, GM-CSF, IL-7); growth factors (EGF,TGF- α, VEGF); adaptive cytokines (IL-2, IL-4, IL-5, IL-13, IL-15, IL-17, sCD40L) and anti-inflammatory cytokines (IL-10, IL-1Rα). Of these only three displayed significant positive correlations with dependence on α4β7 for replication IL-1α, IL-8 and IL-7 (Figure 2.24). IL-7 has been found to induce expression and activation of α4β7 (Cimbro, et al., 2012), while IL-8 has been linked with transient upregulation of α4β7 (Sadhu et al., 1998) and IL-1α is a potent inducer of inflammation and activation in endothelial cells, which may be responsible for the recruitment of α4β7 high cells to the region of initial infection.

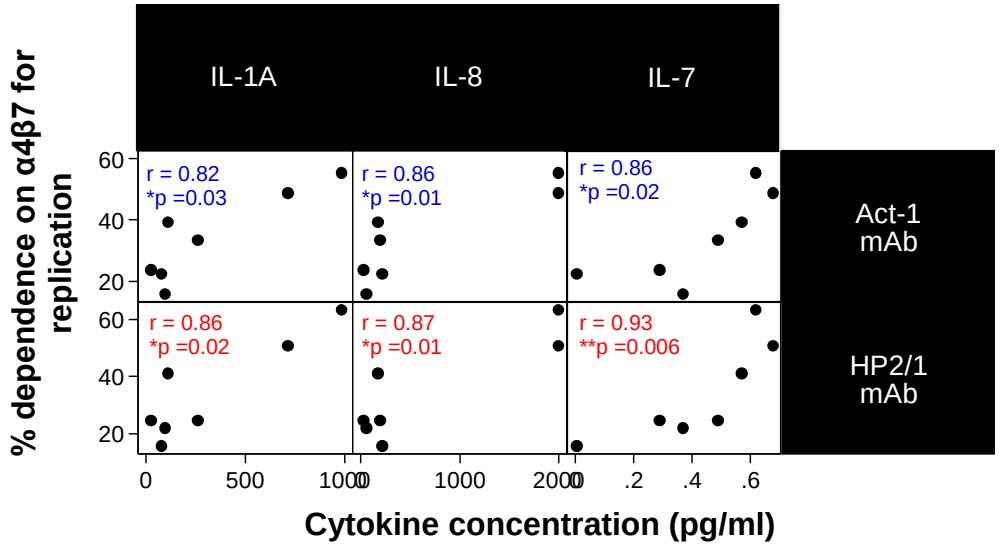


Figure 2.24: Levels of IL-1α, IL-8 and IL-7 in the CVL correlate with dependence on α4β7 for replication at time of HIV transmission
Correlations and significance were calculated using Spearmans coefficient and each dot represents an individual.

Taken together, these data suggest that the environment in which transmission occurs is important to determine whether α4β7 plays a definitive role in transmission.

2.4. Discussion

In this study we investigated the role of α4β7 in HIV pathogenesis and the factors that determine α4β7 reactivity. We show that infectious virus binds α4β7 and that there is a role for α4β7 in viral replication that changes over the course of infection. The dependence on α4β7 for replication was associated with V1/V2 glycan density and loop length and glycans N332, N234 and N334 and in addition CD4 count was negatively associated with α4β7 reactivity. We also found a striking association between the α4β7 binding motif sequence P/SDI/V on the virus and dependence on the integrin for replication as well as diagnosis of bacterial vaginosis in acute HIV infection. Furthermore elevated levels of IL-7, IL-8 and IL-1α in the female genital tract correlated with higher α4β7 reactivity.

While the α4β7 interaction with HIV has been investigated using primarily different gp120 monomers as opposed to infectious and replicative viruses, this study shows that infective viruses bind α4β7 by both a competition as well as a flow cytometry based binding assay. Nawaz and colleagues used CAP88 1.00.17-5A (T/F) and CAP88.12M.2.2F (12 months) to illustrate that gp120s of early transmitting viruses have a higher affinity for α4β7 than the gp120s of chronic viruses. In this study, using infectious viruses from the same individual (CAP88) a similar observation was made. This supports the hypothesis put forward in a recent study that although functional trimers on infectious viruses are likely to be different in conformation to monomers, α4β7 binding is not greatly different in either case (Etemad, et al., 2013). It is important to note that a larger number of trimers on the surface of HIV are non-functional (Moore et al., 2006) and while they may not participate actively in virus entry they are able to elicit potent immune responses and may play a role in α4β7 binding should V1/V2 be exposed optimally. In a recent study, a number of V2 directed mAbs were found to bind epitopes overlapping the α4β7 binding motif and mutations at this site dramatically altered binding affinity of these mAbs (Mayr, et al., et al., 2013). This indicates that this region on the trimer is optimally exposed in order to bind α4β7. In addition, others have submitted that being an attachment factor, the selective advantage of α4β7 in HIV replication would be most prominent at low viral titers

(Parrish, et al., 2012), however we found no difference in α4β7 binding within a range of virus concentrations.

While a recent study investigated differences in α4β7 reactivity and dependence on the integrin for replication between T/F and unrelated chronic subtype C infectious viruses (Parrish, et al., 2012) and another used matched early (not T/F) and chronic infectious subtype A viruses (Etemad, et al., 2013), this is the first study that examines the dependence on α4β7 for viral replication longitudinally within the same individual. A previous study has pointed out that viruses generated using a standard HIV backbone, as in this study, do not allow the contribution of gene products other than Env to the T/F phenotype to be assessed and that overexpression of Env in this model compared to naturally occurring viruses prohibits a meaningful assessment of virus–host cell interactions (Parrish, et al., 2013). While they discuss that these differences may skew biological findings and that full proviral IMCs are more relevant, we observed no difference in α4β7 binding or replication dependence on α4β7 in matched IMCs and IECs suggesting that our model was adequate for this study. In the case of individuals with complete data sets (CAP88, CAP200 and CAP206), a change in α4β7 dependence for replication was clear over time. The T/F virus was highly dependent on α4β7 in these three cases with a notable drop in dependence two months post transmission. While a comparative analysis between the T/F gp160 and the matched 2 or 6 month clone of CAP88, 200 and 206 sequences revealed changes that may explain these differences (a change in the highly conserved region of gp41 which may impact on Env glycoprotein expression, a α4β7 binding motif shift and the addition of a glycan in V5, close to CD4 contact sites respectively), none of these changes were common across participants..

Nevertheless, considering all 11 individuals and all 60 viruses, the shift in importance of α4β7 seems to correlate with the hypothesis that the integrin is necessary for initial viral dissemination to the GALT (Arthos, et al., 2008) and perhaps selected for during transmission. The T/F viruses were significantly more dependent on α4β7 compared to clones from 2 months post infection, by which time the virus has migrated to the target tissue (Brenchley and Douek, 2008) and perhaps α4β7 high affinity viruses are no longer selected for or that many of these replicative competent $CD4^+$ T cells

(Cicala, et al., 2009) are severely and selectively diminished in number. In other studies a small sample of acute subtype A and C virus Envs bound α4β7 with high affinity, and in some cases, later virus strains showed significantly reduced binding (Nawaz et al. 2011), consistent with an early requirement for infection of α4β7-expressing cells that is dispensable once infection in the gut mucosa has been established. Strikingly, there was no significant difference in α4β7 dependence between T/F viruses and chronic clones at 6 and 12 months and this mirrors the finding that there is no difference between T/F viruses and chronic viruses in the first year of infection (Parrish, et al., 2012).

A number of studies have investigated the density of glycans and the variable loop length of V1/V2 as a determining factor of α4β7 reactivity with conflicting outcomes. One study showed that the removal of glycans in this region by mutagenesis was associated with an increased binding affinity for α4β7 (Nawaz, et al., 2011) and another illustrated that shorter, less glycosylated V1/V2 loops do not preferentially bind α4β7 (Etemad, et al., 2013). Results from this study support the latter in that α4β7 dependence was associated with higher glycan density in V1/V2 and longer V1/V2 loop length. While glycans may offer stabilisation to the flexible V1/V2 loop perhaps enhancing α4β7 contact with the region, it is possible that density and length are not as important as the location of the glycans on gp160. This is reflected by the finding that glycans positioned 5 amino acids distal from the α4β7 binding motif are present in a higher frequency in viruses that are highly dependent on α4β7 for replication. Although this observation was not statistically significant, this evidence corroborates the hypothesis that glycans may play a role in α4β7 interaction with gp120.

While others have investigated the role of glycans in V1/V2 on α4β7 binding (Nawaz, et al., 2011; Etemad, et al., 2013), this is the first study to examine the importance of glycans throughout gp160 in this interaction. 9 notable glycans situated throughout gp120 were found to be present at a higher frequency in viruses that have a higher replicative dependence on α4β7. Of these only 2 were present in the V1/V2 region (N150 and N185) which is where the tripeptide α4β7 binding motif is located, and as previously shown (Figure 2.15), linear distance from the motif may impact on affinity of gp120 for the integrin. Interestingly, glycans outside of V1/V2 showed the greatest differences between low

and high dependence on α4β7 for replication. While Nawaz and colleagues showed that removal of N139, N145, and N184 increased α4β7 binding in a subtype C virus, none of these observations were reflected in frequency of these glycans in viruses with high α4β7 dependence. Several other glycans are located near points of CD4 contact with gp120 (N130, N355, N362, N462) which may influence α4β7 binding as CD4 and α4β7 are in such close proximity they can be co-precipitated on the cell surface (Cicala, et al., 2009). Indeed the underrepresentation of N332 and a closely positioned N339 in viruses with a high α4β7 dependence is intriguing as this glycan is diminished in T/F viruses (Moore, et al., 2012) and decreases the fitness of the virus (Moore, unpublished) correlating with the selective advantage α4β7 offers the virus during replication. Over all, these findings suggest that differences in sequences and glycan occupancy in V1/V2 alone cannot explain α4β7 reactivity and its changing role in replication and should not be used in isolation as a signature.

Although viral load was not associated with α4β7 dependence for replication, CD4 count was negatively correlated. This is probably an artefact of the data that were available for specific time points. As can be noted in the Appendix Table B2, a number of transmission time points were not available as the CD4 count was not recorded. As such, the negative correlation reflects the increase in α4β7 dependence at 3 years post infection as previously mentioned. Interestingly, while rapid and intermediate progressors show no differences in α4β7 reactivity, viruses isolated from individuals that go on to produce BCN antibodies show a decreased dependence for α4β7 at transmission as compared to those that do not. While the only clinical outcome that broadly-cross neutralising capacity has been associated with is viral load (Doria-Rose, et al., 2010), and α4β7 dependence is independent of viral load it is possible that BCN individuals select against α4β7 at transmission as a result of their unique immune profile. While intriguing, this requires further investigation.

This study is also the first to illustrate the effect of different minimal α4β7 binding motifs on α4β7 reactivity during replication. As stated previously, α4β7 has three known natural ligands; MAdCAM-1, fibronectin and VCAM-1 arranged in order of strongest affinity for the integrin to weakest (Jackson, 2002). In all of these there is a minimal binding tripeptide motif (LDT, LDV and IDS

respectively) of which the core aspartic acid is pivotal to function (Arthos, et al., 2008). Differences in the dependence on α4β7 for replication between different motifs were evident in viruses isolated from the first year of infection. Intriguingly, the viruses with motifs most closely resembling those of the natural ligand MAdCAM-1 (LDI/L/T) exhibit the lowest dependence on α4β7 for replication. α4β7 is unusual amongst integrins for its ability to mediate both rolling and firm adhesion (Bargatze et al., 1995). The high resolution structure of MAdCAM-1recently reported suggests that it is the unusual flexibility of the integrin binding loop of this ligand that allows for these functions (Yu et al., 2013) not unlike the highly flexible V1/V2 loop of gp120 (McKellan, et al., 2011). The MAdCAM-1 sequence of the integrin-binding CD loop is as follows SVQWRG**LDT**SLGAVQSDT with the minimal binding site for α4β7 shown in bold (Yu, et al., 2013). Within the binding loop downstream is another motif that is responsible for stabilisation of the interaction SDT (Yu, et al., 2013). Not only this, but using random peptidic libraries to generate potent inhibitors of the α4β7 integrin for treatment of gastrointestinal disease, a disulphide-bridged cyclic peptide containing the SDT-tripeptide demonstrated a 10 fold selectivity favouring α4β7 over α4β1 binding and increased potency compared to other inhibitors (Dubree et al., 2002). The antagonists inhibit binding of α4β7 to MAdCAM-1 *in vitro* and lymphocyte accumulation *in vivo*. In addition, a SDV containing antagonist was also highly potent and interestingly more so than an LDT antagonists (Dubree, et al., 2002), mirrored by the observation in this study that viruses with SDI/V motifs have a higher reactivity with α4β7 as compared to LDI/T/V motifs. Viruses with PDI/L motifs were also more dependent on α4β7 for replication than LDT like motifs and this may be as a result of proline bringing the virus into closer contact with α4β7 because of its hydrophobic and compact nature. These data suggests that a possible reason for the wide range of reactivities across individuals is that several viruses have selected for binding motifs that are preferential for α4β7 reactivity. It must be noted that within an individual over time, the α4β7 binding motif is highly conserved. It is rare for motif switching to occur and if it does, it appears to be largely driven by co-infection or super-infection. This suggests that the motif is selected for during transmission.

It has been hypothesised that α4β7 may have a role in transmission, as a result of observed preferential binding of gp120 monomers representative of early infection as compared to chronic infection (Nawaz, et al., 2011). While no difference in dependence on the integrin for replication was shown between T/F and chronic viruses at 6 or 12 months in this and other studies (Parrish, et al., 2012; Etemad, et al., 2013), we showed that the effect that α4β7 has on replication decreases shortly after transmission. Despite this observation, there was a wide range of α4β7 reactivity for T/F viruses (22- 68.8% for HP2/1 and 15.7- 66.9% for Act-1). It is possible that the environment during transmission determines how great a role α4β7 plays during this time, suggesting that there may be a selective advantage for HIV to bind to α4β7. A result of this proposed bottleneck is that T/F viruses expressing P/SDV/I α4β7 binding motifs have significantly higher dependence on α4β7 for viral replication as compared to those expressing LDI/L as previously discussed.

Inflammation in the genital tract has been implicated risk factors for HIV and as such contribute to a distinct bottleneck during transmission (Laga et al., 1993; Mlisana et al., 2012; Stamm et al., 1988). Intriguingly we noted that individuals with T/F viruses with high α4β7 reactivity were associated with BV diagnosis at the time of transmission. BV has been associated with changes in the genital cytokine environment that are distinct from that induced in STI infection inclusive of a mixed inflammatory profile with upregulated pro-inflammatory cytokines (IL-1α, IL-1β and TNF-β) and downregulated chemokines (IP-10, GRO, MDC and MIP-1α) (Ryckman et al., 2008); Masson, et al., in review), The difference in inflammatory profiles from those elicited in STIs is not clear and more importantly the disturbance of vaginal flora characteristic of BV has been associated with HIV acquisition (Taha et al., 1998). Our data may suggest that BV is associated with the transmission of viruses that have a higher dependence on α4β7 for replication by either enhancing the amount of $\alpha4\beta7^+$ T cells in the area of transmission or upregulating α4β7 expression on these cells. This may explain in part why BV is associated with enhanced acquisition and may suggest that α4β7 offers a selective advantage during transmission.

IL-1α, IL-8 and IL-7 in the CVL at the time of transmission were found to correlate significantly with the dependence of T/F viruses on α4β7 for viral replication in our system. IL-8 has been associated with transient upregulation of α4β7 (Sadhu, et al., 1998) and IL-7 has been found to induce expression and activation of α4β7 (Cimbro, et al., 2012) and IL-1α is a potent inducer of inflammation and activation in endothelial cells, which may be responsible for the recruitment of α4β7 high cells to the region of initial infection. While this remains to be confirmed in these data, the spontaneous rise in endogenous IL-7 during the progression to full-blown AIDS and the consequent upregulation of α4β7 may explain the increase in viral replication dependence on the integrin 3 years post infection (Cimbro, et al., 2012). Owing to the small numbers available for analysis it is possible that there are many other cytokines that may be associated with α4β7 reactivity but have been excluded due to lack of significance. As such, it will be important to expand this analysis with the inclusion of more T/F viruses. Cumulatively, this confirms that the environment in which transmission takes place has a big impact on the transmission bottleneck which appears to determine α4β7 reactivity including BV diagnosis and elevated levels of IL-7, IL-8 and IL-1α.

While the importance of α4β7 in HIV transmission and pathogenesis remains controversial in the literature, using a large panel of viruses we determined that the role of α4β7 changes over the course of infection in that shortly after transmission, dependence on α4β7 for replication declines. This correlates with HIV pathology; the virus would have a greater dependence on α4β7 important for wide-spread dissemination during transmission and acute infection, with the need for spread greatly decreased thereafter. We showed that multiple factors may determine high α4β7 reactivity throughout pathogenesis including but not limited to V1/V2 loop length and glycan density, distance of glycans from the α4β7 binding motif, glycan occupancy at positions N234, N334 and N332, low CD4 count and α4β7 binding motif sequence. We also showed that the environment at the site of transmission such as the diagnosis of BV and upregulation of cytokines IL-1α, IL-7 and IL-8 was associated with α4β7 reactivity.

Collectively, these data suggest that there is a role for α4β7 in HIV pathogenesis and that the interaction may be selected for during transmission possibly as a result of a number of factors, one of which appears to be the presence of BV. As a result of the α4β7 binding motif being in a highly immunogenic region of gp120 and antibodies directed against this region associated with protection in the most effective vaccine trial to date, further understanding of the interaction between the virus and the integrin provides an arsenal for the development of future vaccine and therapeutic strategies.

CHAPTER 3

EXPRESSION OF α4β7^{+} ON TH17 AND TREG CD4^{+} T LYMPHOCYTE SUBSETS AND PERMISSIBILITY FOR HIV INFECTION

3.1 Introduction

Regardless of the route of transmission, HIV replicates rapidly in GALT several days after transmission. $\alpha4\beta7^+$ $CD4^+$ T cells have been described as highly permissive to acute HIV and SIV infection as a result of their high activation level and expression of CCR5 (Cicala et al., 2009; Kader et al., 2009; Zhu et al., 2009). Not only are these cells readily infected by HIV and α4β7 is an attachment factor for gp120 (Arthos, et al., 2008) but they are able to home to sites distal from the site of transmission such as the GALT, Peyer's patches, mesenteric lymph nodes and gut lamina propria (Gorfu, et al., 2009; Berlin et al., 1993; Kunkel et al., 2000; Wagner et al., 1996). In support of this route of viral dissemination, $\alpha4\beta7^+$ T cells as well as cells expressing the integrin's putative ligand, MAdCAM-1, are found in the genital mucosa (McKinnon, et al., 2011; Kelly, et al., 2001).

While a number of different T helper subsets have been defined, two of these function in the homeostasis of inflammation. This balance is disrupted in the GALT during HIV infection and this may be the cause of microbial translocation during HIV pathogenesis. These subsets, T helper 17 (pro-inflammatory) and T regulatory cells (anti-inflammatory), are present in the GALT and genital mucosa, are regulated by retinoic acid (Elias et al., 2008; Uematsu et al., 2008) and express α4β7 (Kader, et al., 2009). Th17 cells are involved in the maintenance of mucosal barriers and have been found to be preferentially depleted in acute HIV infection, compromising the integrity of the gut lining and allowing for leaking of lipopolysaccharides and consequent damaging immune activation (Brenchley, et al., 2008). A recent study indicated that Th17 cells express high levels of α4β7, CD4 and CXCR4 but low levels of CCR5 ligands which makes them susceptible to HIV (Alvarez, et al., 2013). Conversely, Treg $CD4^+$ T cells function to attenuate immune responses including Th17 inflammatory effects (Crome, et al., 2010) and have been found to increase in frequency following HIV infection (Chevailer, et al., 2013). Furthermore FOXP3, the defining transcription factor of Treg cells, has been suggested as a marker of disease progression (Suchard, et al., 2010). Both Treg and Th17 phenotypes of $CD4^+$ T cells have been found to support HIV replication (El Hed, et al., 2010; Moreno-Fernandez, et al., 2009).

The founder target cell population of HIV has not been conclusively determined; however it is possible that the virus commandeers the natural homing mechanism to the gut by binding to α4β7, after which it reaches a highly permissive target population. Chapter 2 discusses evidence for a role for α4β7 in HIV pathogenesis and suggests that shortly after transmission, α4β7-dependent infectivity decreases. At this point the virus has already disseminated to the GALT. The phenotype of the α4β7-expressing cells that may support dissemination and their suitability to such a function has not been investigated in the context of HIV.

In this study we sought to determine the effect of all-trans retinoic acid (ATRA) on the frequency of Th17 and Treg $CD4^+$ T subsets in the peripheral blood from healthy donors in order to mimic the GALT environment where dendritic cells secrete ATRA to upregulate homing receptors (Iwata, et al., 2004). We also sought to directly compare the susceptibility of Th17 and Treg $CD4^+$ T subsets to harbour HIV infection as well as their relative expression of the homing receptor α4β7. The comparison between Treg and Th17 cells in this respect is a novel one. These findings along with the natural functions of these subsets may allow for the elucidation of new routes of viral dissemination which can be targeted to prevent the systemic spread of the virus from the genital tract and the establishment of latent reservoirs.

3.2 Materials and Methods

3.2.1 Isolation and stimulation of $CD4^+$ T lymphocytes from whole blood

$CD4^+$ T cells were isolated as described in Chapter 2 from healthy donors from the South African National Blood Bank using the RosetteSep Human $CD4^+$ T cell Enrichment Cocktail (Stem Cell Technologies, Canada) as per the manufacturer's protocol. Cells were cultured in the presence or absence of ATRA (Sigma-Aldrich, Missouri) to upregulate the expression of α4β7 (a gut-homing molecule) and to mimic the GALT environment *in vitro* on the isolated $CD4^+$ T cells in culture. The most physiologically relevant and optimum concentration of ATRA was determined as 10 μM following titration. 50 ng/ml OKT3 (anti-CD3 antibody) (eBioscience, California) and 20 U/ml IL-2 (Roche Applied Sciences, Germany) were added to the $CD4^+$ lymphocytes in RPMI media with 20% FBS and cultured for six days at 37˚C at 5% CO_2. The purity of the $CD4^+$ T cell isolation and the upregulation of α4β7 was confirmed by flow cytometric staining with optimally titrated amounts of CD3-ECD (Beckham Coulter, France), CD4-Qdot 605 (Invitrogen, Carlsbad, CA), Anti-Human CD49d (integrin alpha 4) PE (eBioscience, CA) and anti-human/mouse integrin β7 FITC (eBioscience, CA) and Aqua Fluorescent Reactive Dye (Invitrogen, Molecular Probes, Carlsbad) was used to monitor cell viability. The gating strategy was as indicated in Figure 2.1 in Chapter 2. The samples from the donors were designated responders and non-responders to the ATRA treatment based on the increase or no effect, respectively, of the ATRA treatment on the $\alpha4\beta7^+$ population of lymphocytes.

3.2.2 Th17 and Treg phenotyping of $CD4^+$ T cells by flow cytometry

In order to determine the nature of the subset on which α4β7 is prominent, Th17 and Treg subsets, were investigated. Surface staining was performed as described above, while cells for intracellular staining for FOXP3 and IL-17 was initially activated with phorbol 12-myristate 13-acetate (PMA) at 10 ng/ml, Ionomycin (500 ng/ml) and Brefeldin A (10 μg/ml) for a period of 4 hours. Unstimulated cells were used to determine the level of background staining. Both PMA and ionomycin were used to

activate the T cells in order to stimulate the production of the chemokine IL-17. Fluorochromes were diluted in a 1% BSA/ PBS buffer up to a staining volume of 50 µl. 50 µl of cells were then added to the staining mixture and incubated in the dark for 30 minutes at 4 ˚C. Staining was then completed as per the manufacturer's protocol (One-step protocol for intracellular (nuclear) protein, eBioscience) and the FOXP3 Staining Buffer Set (eBioscience, San Diego) was used to provide the optimum buffering system for staining of the FOXP3 transcription factor. For cell surface staining, CD195/CCR5 PE-Cy5 (BD Pharmingen, San Diego, CA), CD3-ECD (Beckham Coulter, France), CD4-Qdot 605 (Invitrogen, Carlsbad, CA), anti-human CD49d (Integrin alpha 4) PE (eBioscience, CA) and anti-human/mouse integrin β7 FITC (eBioscience, CA), mouse anti-human CD196/CCR6 PerCP-Cy5.5, mouse anti-human CD45RA APC-H7, CD25-AF700 (BD Biosciences), CD127-PE-Cy7 (BD Biosciences) were used. Intracellular markers included FOXP3-V450 (BD Horizon) and IL-17-AF642 (BD Biosciences). Following staining, cells were fixed in 0.1% paraformalydehyde in PBS. Th17 $CD4^+$ T cells were delineated as $CCR6^+$ $IL\text{-}17^+$ $CD4^+$ T lymphocytes, as shown in Figure 3.1. Treg $CD4^+$ T cells also shown in Figure 3.1 were of $CD25^{high}$ (gated on the highest 2% of the $CD25^+$ $CD4^+$ population) $FOXP3^+$ $CD127^-$ $CD4^+$ T lymphocytes. Both defined populations were analysed for expression of α4β7, CCR5, CD45RA. In addition $IL\text{-}17^+$ and $FOXP3^+$ populations were analysed for the expression of α4β7, CCR5, CD45RA as a consequence of the controversial nature of defining the Th17 and Treg T subsets. α4β7+ CD4+ T cells were also assessed for Treg, Th17 and IL-17/FOXP3 expression indicated by the dashed arrows in Figure 3.1. Acquiring of all samples was performed on a FACSAria (BD Biosciences). All data analysis was done on FlowJo X software (TreeStar Ashland, OR). 100000 events were acquired in each experiment.

3.2.3 Flow sorting of $CD4^+$ T lymphocytes

In order to test the permissibility of $\alpha4\beta7^+$ populations to infection, ATRA treated $CD4^+$ T cells were stained as previously described (Chapter 2 Section 2.2.3) and sorted on the FACSAria into four populations (i) $\alpha4^-\beta7^-$ (ii) $\alpha4^-\beta7^+$ (iii) $\alpha4^+\beta7^-$ and (iv) $\alpha4^+\beta7^+$ $CD3^+$ $CD4^+$ T cells. These populations (at 4×10^6 cells/ml) were then infected with a transmitted/founder (T/F) infectious envelope clone (IEC) (CAP88.2.00.17-5A) produced as discussed (Chapter 2 Section 2.2.4) and following a 2 hour

incubation at 37˚C and 5% CO_2, the cells were washed twice with 1% BSA/PBS. The cells were harvested over 10 days into 1.25% empigen, and a p24 ELISA was done as discussed in Chapter 2 Section 2.2.4 to measure HIV infection.

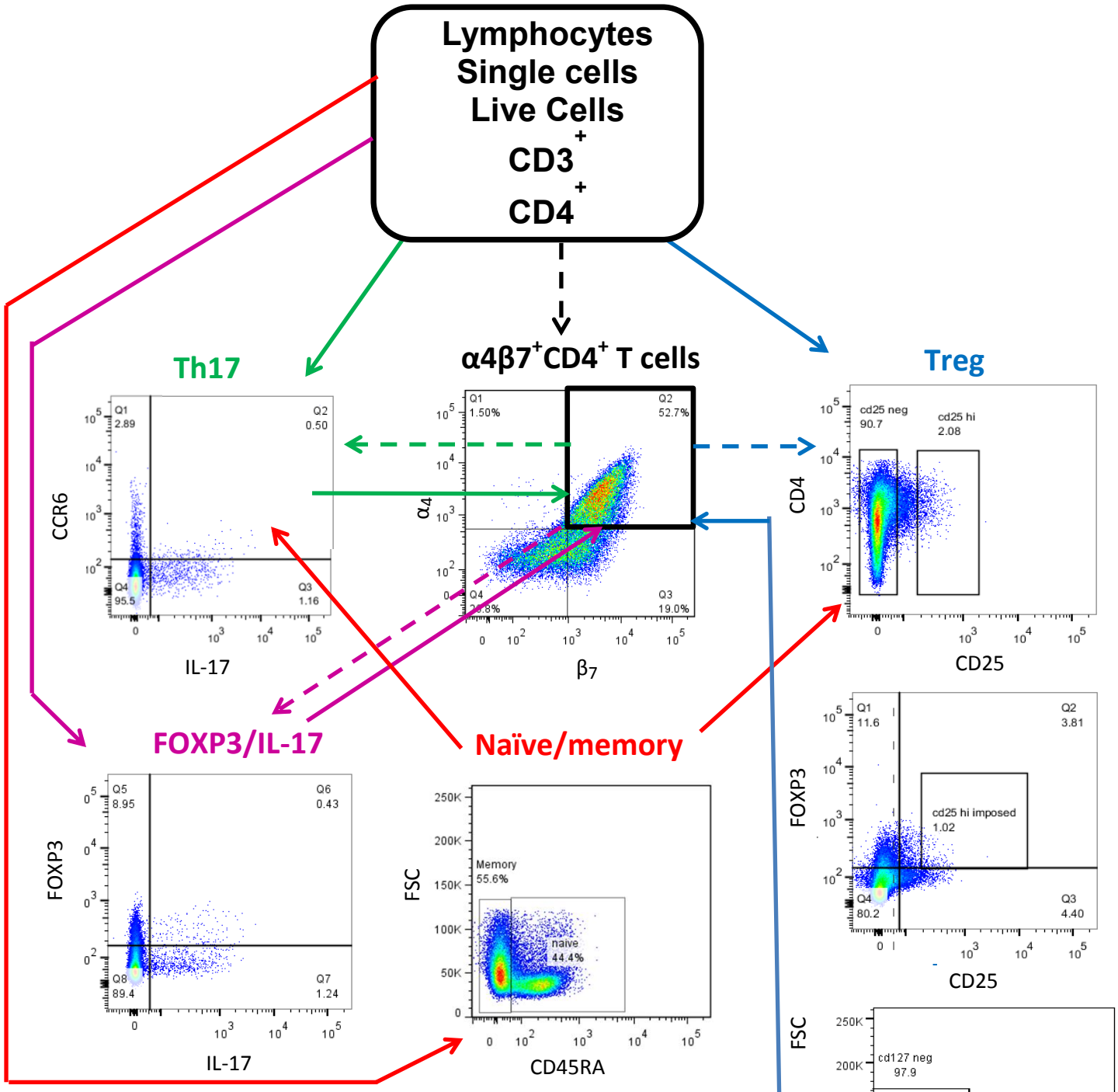


Figure 3.1: Gating strategies for Th17, Treg, IL-17 or FOXP3 expressing α4β7^{+}CD4^{+} T cells

Dashed arrows indicate α4β7^{+} T lymphocytes gated as α4β7^{+} CD4^{+} CD3^{+} live single lymphocytes (black box) and then defined as Th17 cells (green arrows) based on dual expression of CCR6 and IL-17. The Treg subset was defined as CD25^{high} FOXP3^{+} CD127^{-} population (blue arrows) and IL-17 and FOXP3 expression (purple arrows) was also determined. The solid arrows show those populations gated on CD4+ T lymphocytes. Treg, Th17, IL-17/FOXP3 and naïve CD4+ T cells were then gated for α4β7 while CD45RA- and memory CD45RA+ (red arrows) CD4 T cells were gated for Th17 and Treg and then assessed for α4β7 expression.

3.2.4 Permissiveness of α4β7$^+$ Treg and Th17 CD4$^+$ subsets to HIV infection

Following the isolation of CD4$^+$ T cells from healthy donors and culturing with ATRA as previously described (Chapter 2 Section 2.2.2), the lymphocytes were infected with a T/F IEC (CAP88. 2.00.17-5A) as previously described (Chapter 2 Section 2.2.6). Subsequently, after three days of incubation at 37˚C, the cells were washed and resuspended in 1% BSA/PBS and stained to denote Th17 and Treg subsets and their α4β7 expression as previously described. In addition, viral replication was noted by intracellular p24 staining (following the same intracellular staining protocol as per the manufacturer of eBiosciences FOXP3 intracellular staining kit), with Act-1, HP2/1, CD4 and uninfected controls. This extensive panel enabled us to confirm how the expression of Treg and Th17 markers and α4β7 are affected on HIV infected cell populations as well as the comparative level of p24 in these cells. This enabled us to investigate the impact of HIV infection on Treg and Th17 CD4$^+$ T cells and the effect of α4β7 expression in these subsets.

3.2.5 Statistics

The Wilcoxon matched-pairs signed rank t test or paired t test was performed on paired sample comparisons while independent sample comparisons were done using the Mann-Whitney test of Friedman's test. $p > 0.05$ was used to define a significant observation.

3.3 Results

3.3.1 ATRA changes the phenotypic profile of CD4$^+$ T cells

CD4$^+$ T lymphocytes from healthy donors were incubated with ATRA at varying concentrations (1 µM, 100 nM, 10 nM) to optimise upregulation of the α4β7 integrin, a marker of the gut-homing phenotype. In line with previous studies (Arthos, et al., 2008; Kang, et al., 2011), 10 nM, a concentration similar to that found at physiological conditions in the GALT, was the most effective at upregulating α4β7 expression represented in Figure 3.2 by the greatest shift in β7-FITC fluorescence (yellow) compared to ATRA untreated cells (grey).

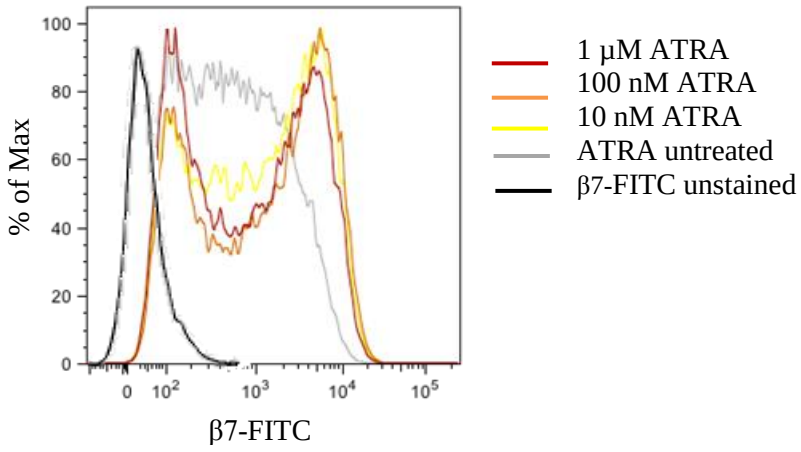


Figure 3.2: Determination of optimal ATRA concentration for activation of CD4$^+$ T lymphocytes
ATRA concentrations of 1 µM, 100 nM and 10 nM are indicated in yellow, orange and red respectively with the untreated control displayed in grey and the staining control in black. 10 nM showed the greatest upregulation of α4β7. CD4$^+$ live T lymphocytes were gated for α4β7 and increases in α4β7 represented by increases in β7-FITC fluorescence.

Interestingly, approximately 25% of donors responded to ATRA treatment in terms of the upregulation of α4β7 as shown in Figure 3.3 in line with findings from others (Arthos, et al., 2008). Only those individuals that responded in this way were included in this study. Untreated non responders and responders exhibited a mean expression of 33.19 ± 2.91 % and 31.33 ± 2.94 % respectively. ATRA treatment of responders increased α4β7 expression significantly (Paired t test at 95% confidence interval (c.i.) by 20% to a mean of 50.43 ± 3.48%.). Results show that in response to ATRA treatment, there was an upregulation in α4β7 expression on CD4$^+$ T cells (number per cell

represented by median fluorescence intensity) and an increase in frequency of α4β7 expressing $CD4^+$ T cells (Figure 3.4A and B). This is not the only receptor that comes into contact with HIV that is affected by ATRA treatment. CCR5 expression is also increased significantly (Figure 3.4C) on the surface of $CD4^+$ T cells. This, along with α4β7 upregulation may explain in part why $CD4^+$ T cells that are treated with ATRA show higher levels of infectivity over ten days than those that are not (Figure 3.4D).

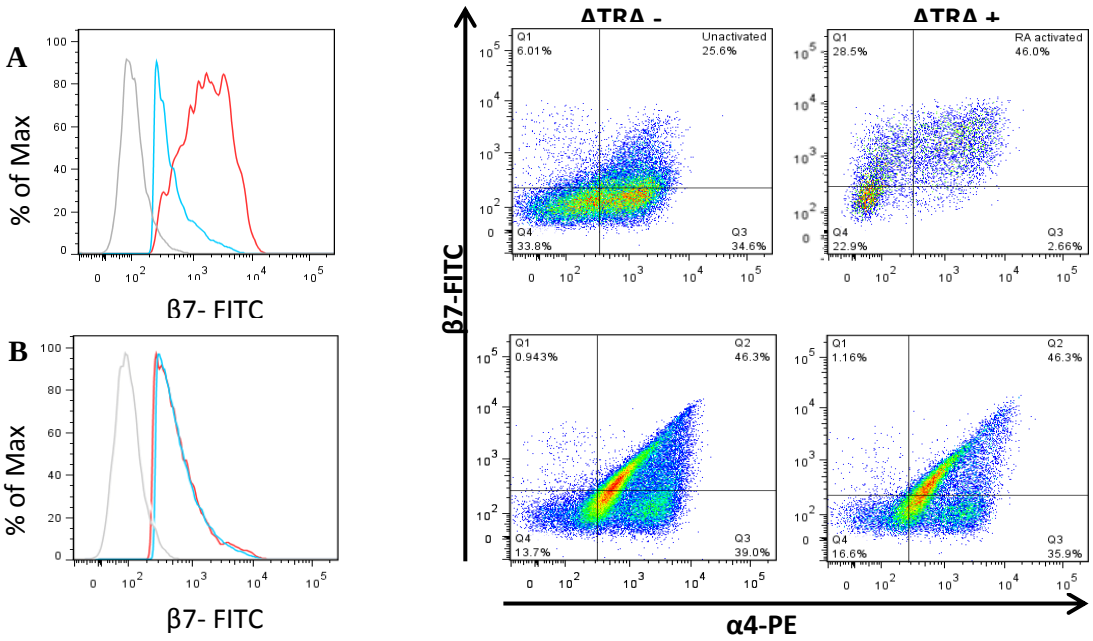


Figure 3.3: Characterisation of a Responder and Non-Responder to ATRA Treatment
CD4 cells were treated with ATRA for 6 days and stained for β7. Red lines represent ATRA treated samples, blue lines represent untreated controls with an FITC- unstained control displayed in grey. An example of a responder is shown in Panel A and a non-responder in Panel B.

Treg $CD4^+$ T cells (defined in this study as $CD25^{high}$ $FOXP3^+$ $CD127^-$ were increased in frequency following treatment with ATRA represented in terms of absolute numbers of the total lymphocyte population (Figure 3.5A). To corroborate our gating strategy, we also investigated the level of expression of FOXP3 (the master transcription regulator of this subset) and the number of $FOXP3^+$ $CD4^+$ T cells and found that this marker is also upregulated by ATRA. Conversely, Th17 (defined by $CCR6^+$ $IL\text{-}17^+$ expression as in Singh, et al., 2007) and $IL\text{-}17^+$ $CD4^+$ T cells were not significantly affected by ATRA treatment (Figure 3.5C). Ionomycin/PMA treatment is required to stimulate cells to release large amounts of IL-17 while very low background levels were noted in unstimulated cells (Figure 3.5B).

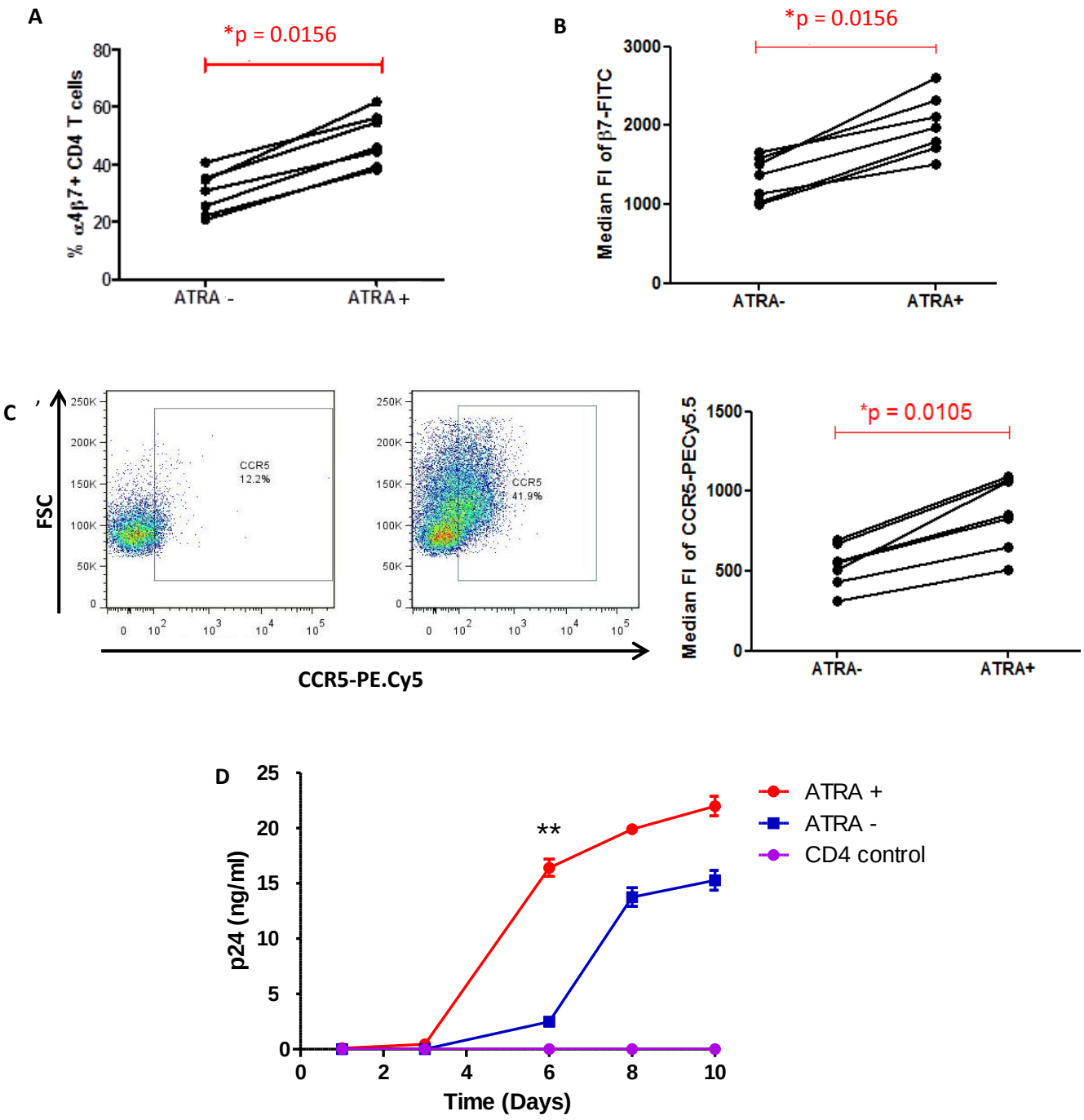


Figure 3.4: ATRA upregulates α4β7 and CCR5 expression on α4β7$^+$ CD4$^+$ T cells and enhances permissibility to HIV infection

ATRA significantly upregulates **(A)** α4β7 expression on CD4$^+$ T cells (p=0.0156, Wilcoxon matched-pairs signed rank test; n=7) and **(B and C)** CCR5 expression on α4β7$^+$ CD4$^+$ T cells (p=0.008, Wilcoxon matched-pairs signed rank test; n=7). Replication of HIV is enhanced by ATRA treatment of CD4$^+$ T cells **(D)** Detection of p24 antigen occurs more rapidly and to higher levels in the ATRA treated cultures (red line) compared to untreated cultures (blue line) and CD4 inhibition control (purple) over 10 days of culture. The difference in infection is significant at day 6 (p = 0.004, paired t test). This figure is representative of four independent experiments with three replicates at each time point.

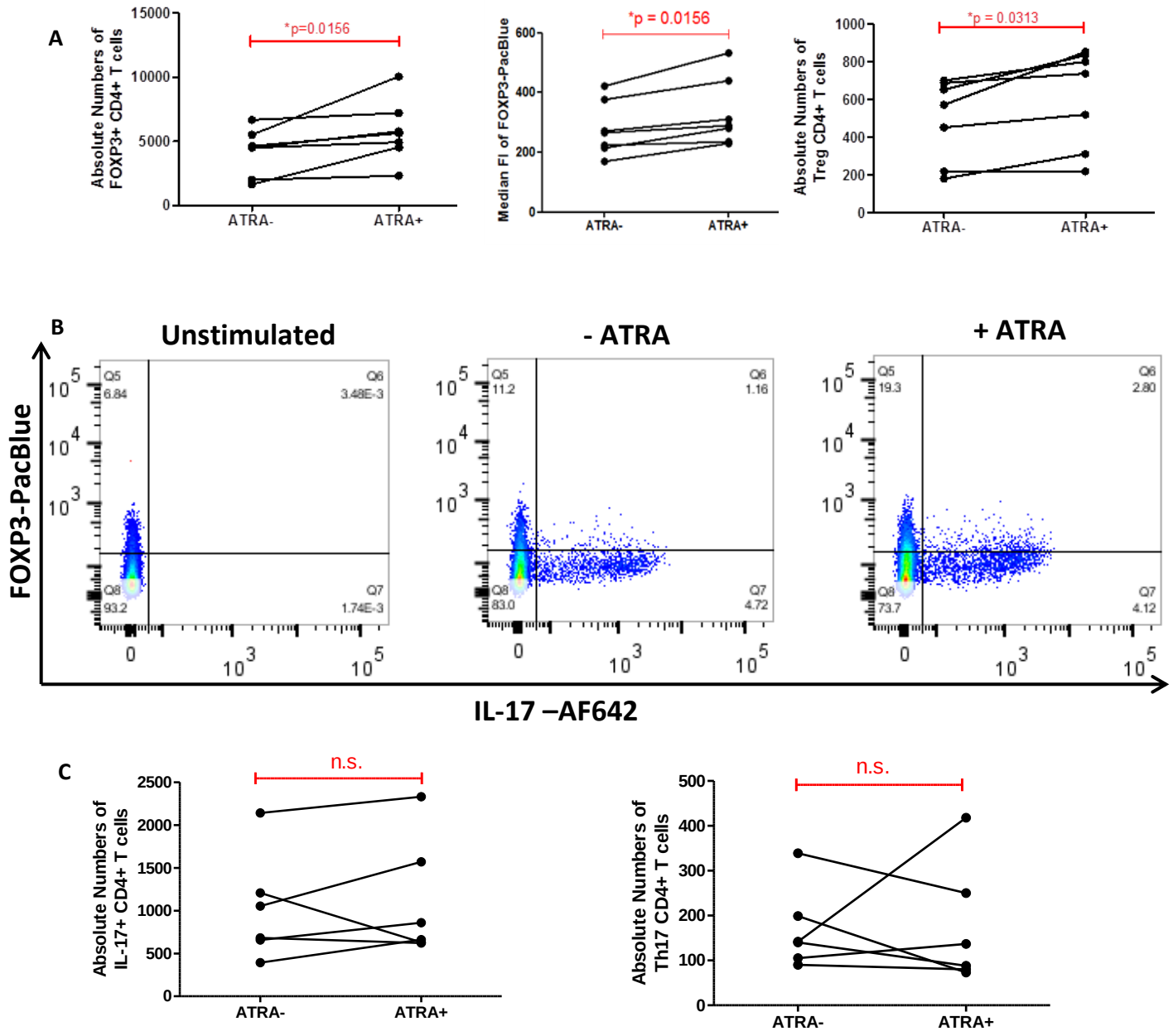


Figure 3.5: ATRA upregulates FOXP3 expression and Treg frequency but has little effect on the Th17 phenotype

(A) ATRA significantly upregulates FOXP3 (MFI determined on FOXP3+ CD4+ population) and the Treg phenotype ($CD25^{high}$ $FOXP3^{+}$ $CD127^{-}$ $CD4^{+}$ T cells) as gated on $CD4^{+}$ T cells as indicated by absolute numbers of cells. **(B)** Representative plot of IL-17 and FOXP3 gated on $CD4^{+}$ T cells showing cells unstimulated with PMA and ionomycin and stimulated cells untreated and treated with ATRA respectively. **(C)** Th17 cells defined as $CCR6^{+}$ $IL17^{+}$ $CD4^{+}$ T lymphocytes and $IL\text{-}17^{+}$ T lymphocytes were not significantly affected by ATRA treated. ATRA treated and untreated matched individuals were compared by Wilcoxon matched-pairs signed rank t test; (n=7) with n.s. referring to non-significant where $p>0.05$.

3.3.2 Treg and Th17 CD4$^+$ T cells express high levels of α4β7

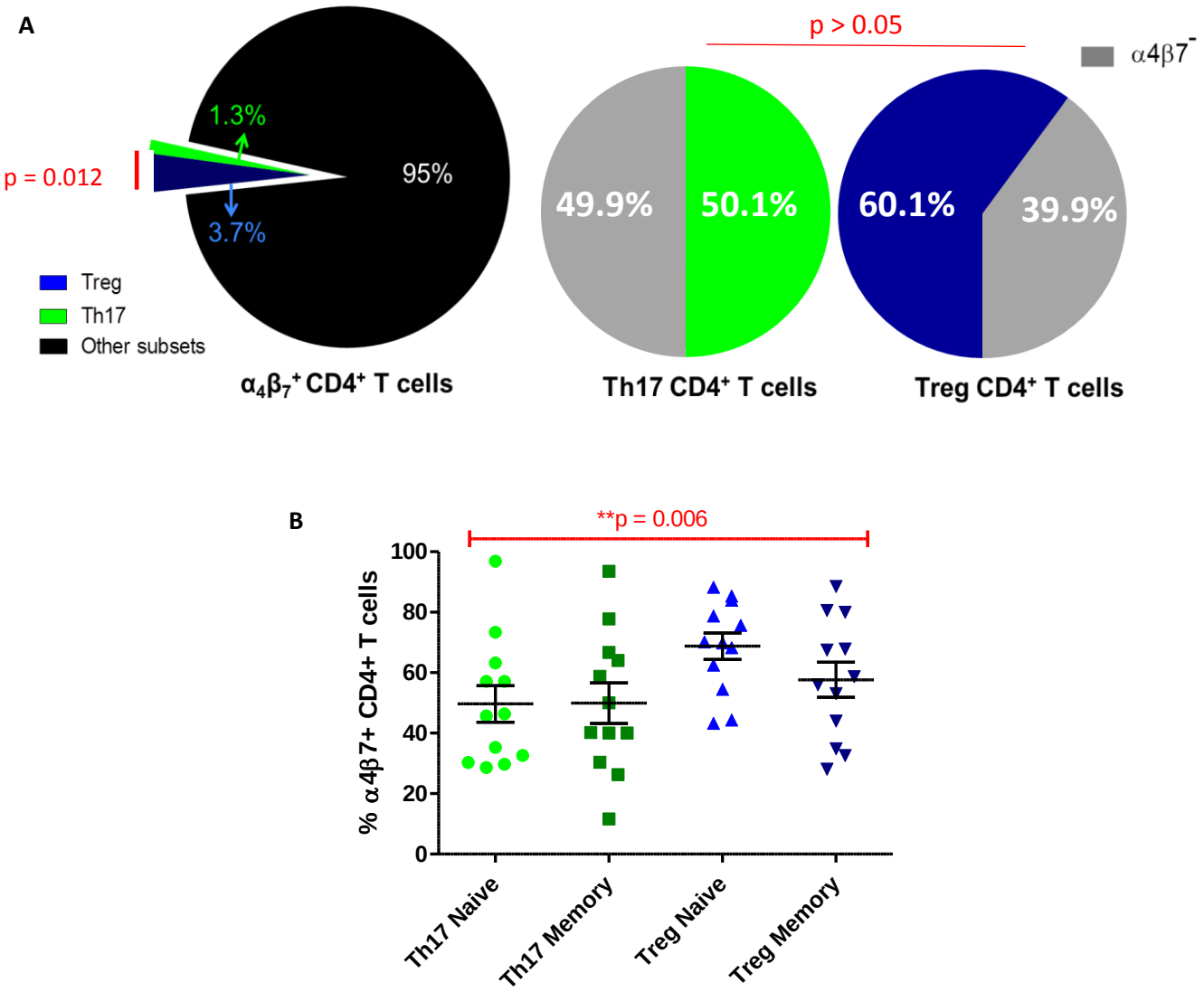


Figure 3.6: Phenotypic characteristics of α4β7$^+$ CD4$^+$ T cell subsets from healthy donors
(A) The α4β7$^+$ CD4$^+$ T subset, shown in the first pie chart consists of other CD4 subsets (black), and a small percentage comprising Treg (blue) and Th17 (green) CD4$^+$ T cells. In this population, the proportion of Treg CD4$^+$ T cells is significantly higher than Th17 CD4$^+$ T cells (p=0.012, Paired t test; n=12). α4β7 is expressed to similar levels on Th17 and Treg CD4$^+$ T cells as shown by the green and blue portions of the second and third pie charts (p>0.05,paired t test; n=12) respectively. The grey portions represent the proportion of Treg or Th17 CD4$^+$ T cells not expressing α4β7. **(B)** α4β7 expression was gated on naïve or memory Treg or Th17 CD4+T cell populations and was highest on the naïve Treg CD4+ T cell population (p=0.006; repeated measures one way ANOVA; n=12).

In a number of defined responders treated with ATRA (mimicry of the GALT environment), the expression of α4β7 on Th17 and Treg subsets was determined to assess the potential homing ability of these cells to the gut. Sixty percent of Treg CD4$^+$ T cells were found to express α4β7 and form a larger proportion of α4β7 CD4$^+$ T cells as compared to Th17 cells (Figure 3.6A). This further suggests

that ATRA favours a Treg phenotype and that both Th17 and Treg subsets are able to home to the GALT. When the memory marker CD45RA was used to discriminate between naïve and memory T cells, the Treg naïve $CD4^+$ T cell population was found to express significantly more α4β7 than other populations (Figure 3.6B). This suggests that this phenotype maybe particularly well suited to home to and attach to HIV preferentially allowing for wide-spread dissemination shortly after transmission.

3.3.3 $\alpha4\beta7^+$ $CD4^+$ T cells are highly permissive to infection

While many have described that α4β7 defines a subset of cells that is highly permissive to HIV infection, it has been based on infection in culture (Cicala, et al., 2009). We sought to determine whether $\alpha4^+\beta7^+$ sorted subsets were more permissive to infection in pure populations. We sorted $CD4^+$ T cells into (i) $\alpha4^+\beta7^-$ (ii) $\alpha4^+\beta7^+$ (iii) $\alpha4^-\beta7^+$ and (iv) $\alpha4^-\beta7^-$ populations and infected them with CAP88 T/F IEC and monitored p24 over 10 days (Figure 3.7 A). Not only was the $\alpha4^+\beta7^+$ population (shown in red) more permissive to infection than the other α4β7 populations, but was more permissive to infection than the unsorted $CD4^+$ T cell population (shown in black) (Figure 3.7B). CCR5 has often been used to define HIV permissive subsets of cells as it is an essential receptor for HIV replication (Samson et al., 1996). $\alpha4\beta7^+$ $CD4^+$ T cells also express significantly higher levels of CCR5 than $\alpha4\beta7^-$ $CD4^+$ T cells (Figure 3.7C) which may explain why this subset is highly permissive to HIV infection.

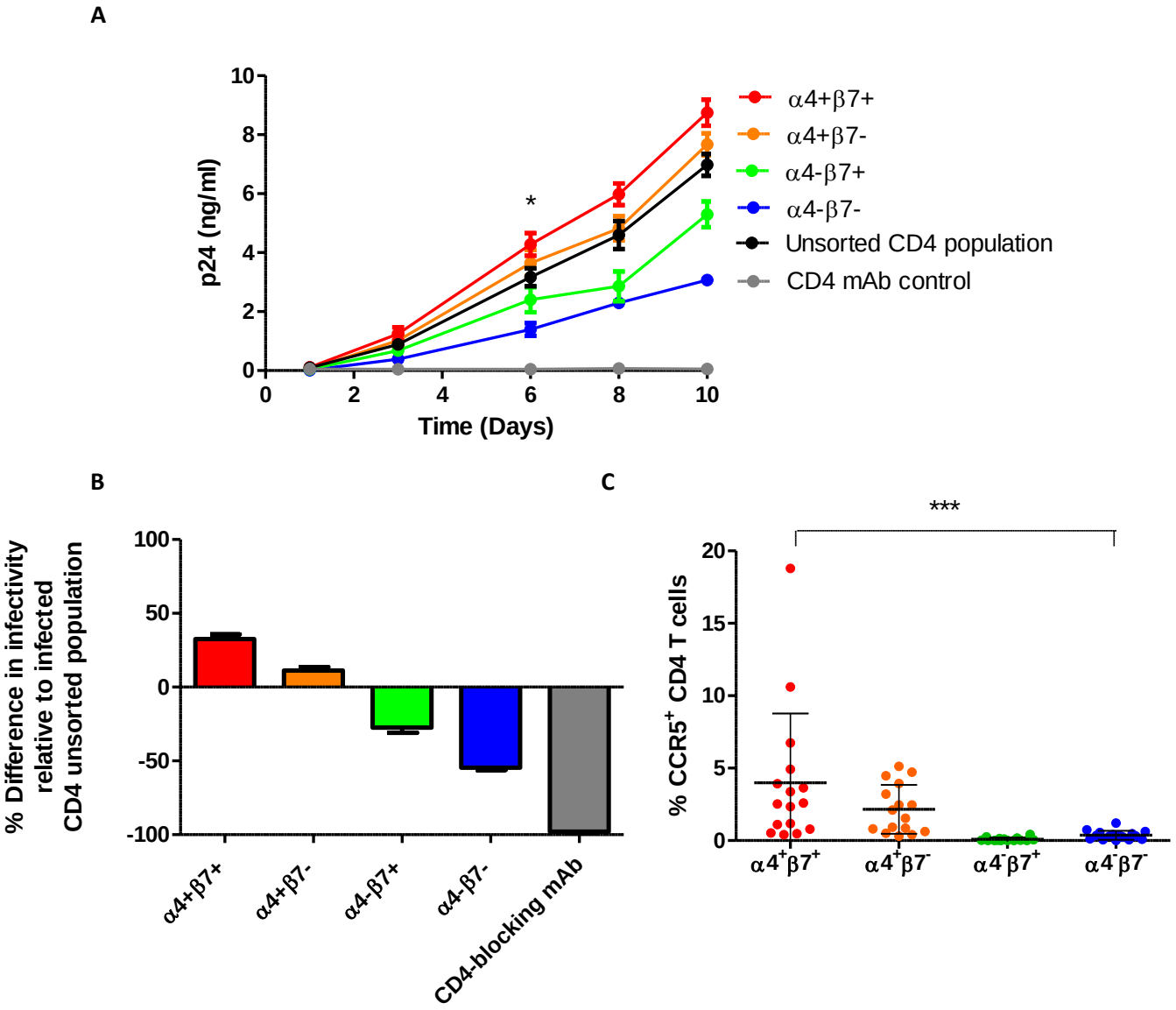


Figure 3.7: α4β7$^+$ CD4$^+$ T lymphocytes are significantly more permissive to HIV infection than α4β7$^-$ subsets

(A) α4$^+$β7$^+$ / α4$^+$β7$^-$/ α4$^-$β7$^+$ and α4$^-$β7$^-$ CD4$^+$ T lymphocytes were sorted by flow cytometry and following infection with CAP88 T/F α4$^+$β7$^+$ (red) shows higher p24 than α4β7$^-$ subset (blue) (significant at day 6 *p <0.01;Friedman test adjusted for multiple comparisons using Dunn's test) with a CD4 control indicating blocking of infection (grey). p24 levels were determined by in-house p24 ELISA. This is representative of 3 independent experiments with 4 replicates in each. An unsorted population of CD4$^+$ T cells was also infected (black) and its permissiveness to infection compared to sorted populations as in **(B)**. Percentage difference in infectivity was calculated as the difference in p24 between the sorted population and CD4$^+$ unsorted population as a proportion of the latter. **(C)** CCR5 expression was significantly higher on α4β7$^+$ as compared to α4β7$^-$ CD4$^+$ T cells (p<0.0001,repeated measures one way ANOVA with Tukey multiple comparisons post test; n=16).

3.3.4 Treg and Th17 CD4$^+$ T cells are highly permissive to acute infection

In order to determine the permissibility of Treg and Th17 subsets to HIV infection under GALT conditions, CD4$^+$ T cells isolated from healthy donors that responded to ATRA by upregulation of α4β7 were infected with CAP88 T/F and subjected to p24 intracellular staining (Figure 3.8B) and Th17/Treg CD4$^+$ phenotyping after 3 days. As CD4 was rapidly downregulated following infection as noted in Parrish, et al., 2012 and in Figure 3.8 A, populations were not gated on this marker but were gated on CD3$^+$ T cells as a representation of CD4$^+$ lymphocytes. As these cells were 98-99% pure for CD4 lymphocytes after CD4 enrichment (data not shown), this gating strategy is unlikely to affect results.

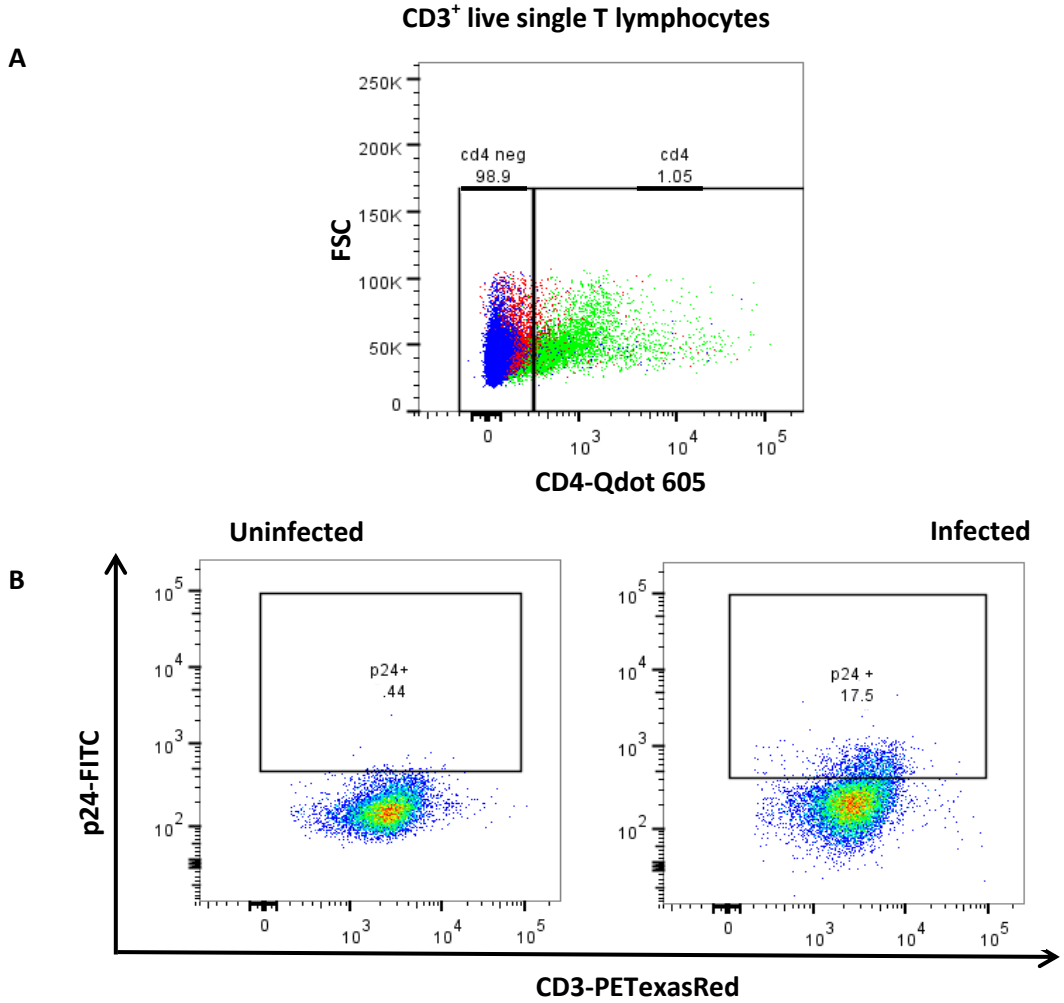


Figure 3.8: Alteration in gating strategy to define infected Th17 and Treg T lymphocytes
(A) CD4 is downregulated on the surface of purified CD4 T lymphocytes following infection with HIV and as a result all subsets were gated on the CD3$^+$ T cell subset alone. The blue population shows the CD3$^+$ T lymphocytes incubated with anti-CD4 mAb, while red is the infected cells and green the uninfected cells. (B) p24 was stained for and was clearly enhanced in the infected samples (right panel) as compared to the uninfected samples (left panel).

The gating strategy was as per Figure 3.1 for Th17 and Treg subsets with p24+ cells serving as the third possible parent gate (shown Figure 3.10B). In some cases where specified, p24+ cells were gated from Treg or Th17 parent population (Figure 3.10 C) or from the Treg α4β7+ or Th17 α4β7+ populations (Figure 3.10 E).

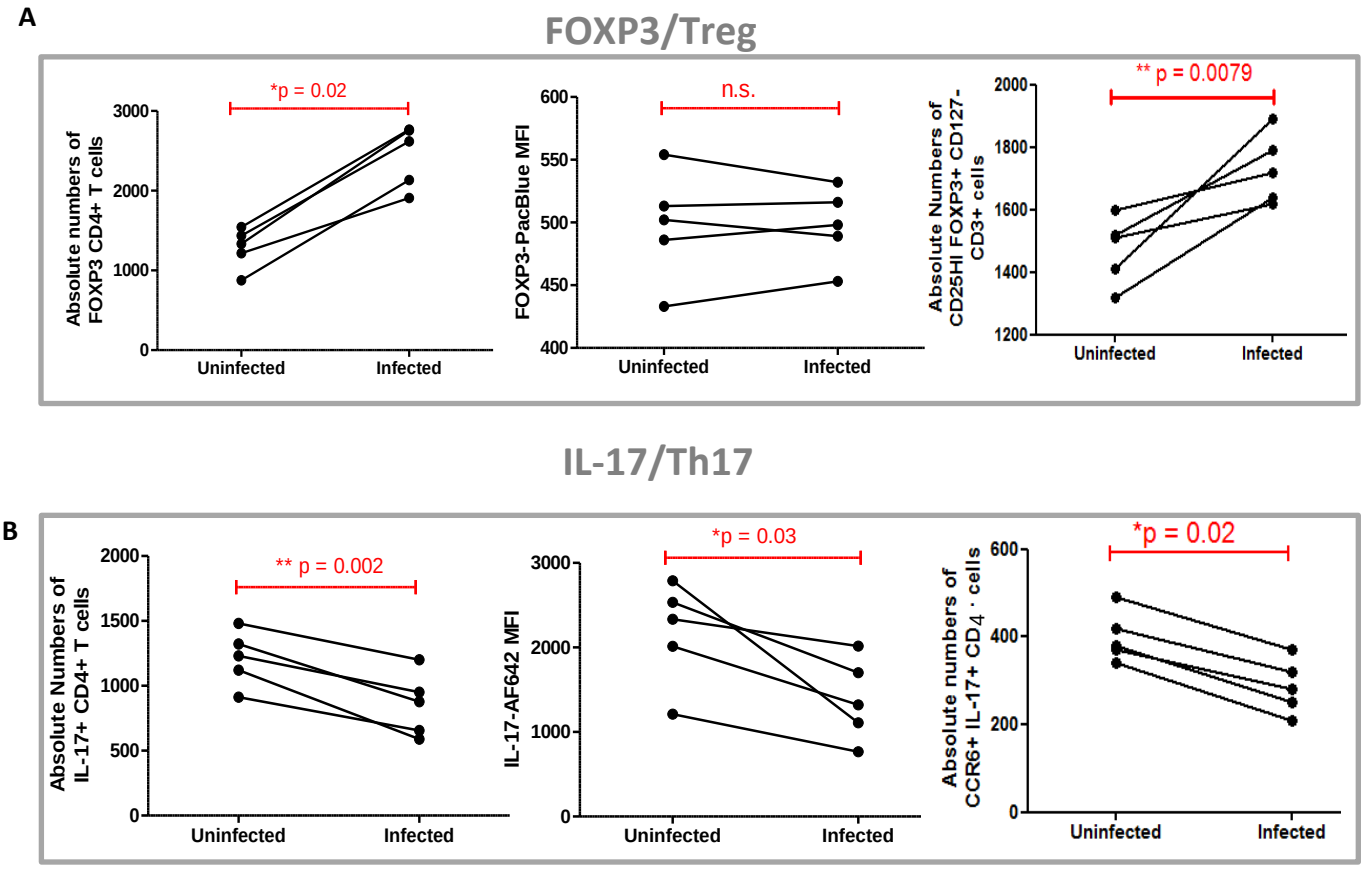


Figure 3.9: Effect of HIV infection on Treg and Th17 phenotypes
(A) FOXP3$^+$ CD4$^+$ T cells (p = 0.02) and Treg CD4$^+$ T cells (CD25^{high} FOXP3$^+$ CD127$^-$) (p =0.008) in the first and last panel respectively were significantly increased in number following infection. FOXP3 expression (MFI determined on FOXP3+ CD4+ T cells) is unchanged following infection (middle panel). HIV infection decreases **(B)** Th17 marker IL-17 expression (p = 0.03), the frequency of IL-17 expressing cells (p = 0.002) and Th17 (CCR6$^+$ IL-17$^+$) T cells (p = 0.02) were decreased following infection. All statistical comparisons were done with a Wilcoxon matched-pairs signed rank t test where n=5

Five individuals were used to examine the effect of HIV infection on the frequency of Th17 and Treg subsets. Treg CD4$^+$ T cells were upregulated and we also confirmed that FOXP3$^+$ (the master regulator of Treg subsets) CD4$^+$ T cells were also increased in number. The median fluorescence intensity of the FOXP3 marker did not change during HIV infection *in vitro*, an indication that expression itself was not affected (Figure 3.9.A). Interestingly, the frequency of Th17 and IL-17$^+$ CD4$^+$ T cells decreased 3 days post infection as did IL-17 expression per cell (Figure 3.9B). This

suggests that not only are these cells depleted, but those that remain have decreased IL-17 expression. This rapid decline has been noted *in vivo*, with data suggesting that Th17 cells are the most prominently depleted $CD4^+$ T cell subset following HIV/SIV infection (Brenchley, et al., 2008). In addition, Treg cells have shown to be increased in number following HIV infection (Li, et al., 2011; Shaw, et al., 2011). The observation that FOXP3, a regulator of Treg function, is unchanged after three days of incubation with virus is in line with other studies that show that suppressive function of Treg cells is maintained following infection (Angin et al., 2012; Kared et al., 2008).

In addition, Treg ($CD25^{high}$ $FOXP3^+$ $CD127^-$ $CD4^+$ T cells) were found to be highly permissive to HIV infection with 71.15% of this population found to be p24 positive (Figure 3.10C), with a significantly higher proportion of $p24^+$ $CD4^+$ T cells being Treg cells as compared to Th17 cells (Figure 3.10B). Despite the significant differences in infectivity, Th17 and Treg $CD4^+$ T cells express similar levels of CCR5 (Figure 3.10D) but as described (Chapter 3 Section 3.3.2), Treg $CD4^+$ T cells express slightly higher levels of α4β7 than Th17 $CD4^+$ T cells although this was not significant. There may be other susceptibility markers that were not investigated that may explain these differences. Interestingly $\alpha4\beta7^+$ Treg and $\alpha4\beta7^+$ Th17 $CD4^+$ T cells show similar levels of infectivity 3 days post infection (Figure 3.10E) despite the fact that Th17 $CD4^+$ T cells express significantly higher levels of CCR5 compared to Treg $CD4^+$ T cells (Figure 3.10F). Act-1 and HP2/1 mAbs that target the α4β7 integrin were able to partially inhibit HIV infection of CAP88 T/F as shown in Chapter 2 and CD4 abrogated HIV replication (Figure 3.10A) confirming the dependence on the integrin for viral replication.

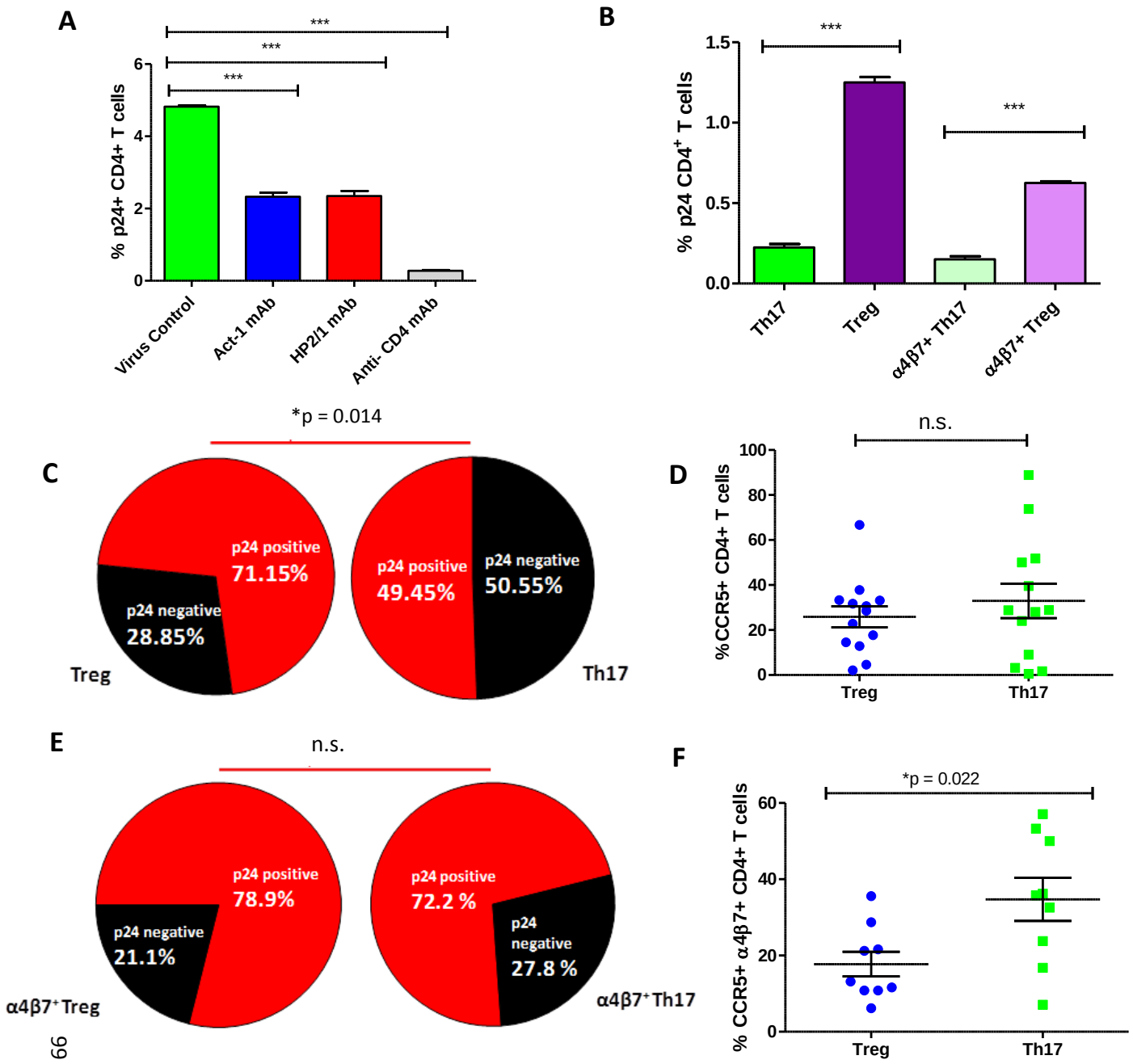


Figure 3.10: p24 levels in Treg and Th17 CD4$^+$ T lymphocytes

(A) CD4$^+$ T lymphocytes were incubated with CAP88 T/F virus and HP2/1, Act-1 and anti-CD4 mAbs and p24 levels compared to virus control Infectivity is expressed as % p24$^+$ CD4$^+$ T cells (Repeated measures one way ANOVA, pairwise comparisons adjusted for by Tukey's method) **(B)** Of the total p24$^+$ CD4$^+$ population both Treg and α4β7$^+$ Treg T lymphocytes make up a larger proportion of cells compared to Th17 cells and α4β7$^+$ Th17 cells respectively (***p<0.0001, paired t test). **(C)** Treg CD4$^+$ T cells express significantly higher p24 levels than Th17 cells three days p.i. (p=0.014; paired t test) but **(E)** show similar levels of infection when gated relative to α4β7 where n.s.= non-significant (p > 0.05;paired t test) and n = 5. **(D)** CCR5 expression was similar on Treg and Th17 CD4$^+$ T cells (p>0.05, paired t test; n=16) and **(F)** CCR5 expression is significantly higher in α4β7$^+$ Treg CD4$^+$ T cells compared to α4β7$^+$ Th17 CD4$^+$ T cells (p=0.022, paired t test; n=9).

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3.4 Discussion

In this study we describe the phenotypic properties of Treg and Th17 $CD4^+$ T subsets that express high levels of α4β7. Studies have shown that dendritic cells in the GALT secrete all trans retinoic acid (ATRA) which upregulates α4β7 on $CD4^+$ T cells (Iwata, et al., 2004). In this study, we describe how ATRA and therefore the GALT environment may influence the phenotype of these subsets irrespective of α4β7 upregulation; with the number of Treg and $FOXP3^+$ $CD4^+$ T cells increasing with ATRA treatment and Th17 and IL-17^+ $CD4^+$ T cell number remaining unchanged. Expression of FOXP3, α4β7 and CCR5 were upregulated by ATRA treatment as was the permissiveness of infection. In addition, Treg $CD4^+$ T cells express slightly more α4β7 than Th17 $CD4^+$ T cells. $\alpha4\beta7^+$ and Treg $CD4^+$ T cells were highly permissive to infection by a T/F virus.

Understanding the effect that ATRA has on Treg and Th17 subsets and the markers CCR5 and α4β7 may elucidate their role in the GALT and their possible involvement in HIV infection. The upregulation of α4β7 and other homing receptors like CCR9 by retinoic acid produced by GALT dendritic cells has been well documented (Iwata, et al., 2004; Arthos, et al., 2008) and was confirmed in this study using ATRA. CCR5 was also upregulated on $CD4^+$ T cells as a whole as well as on Treg and Th17 subsets (data not shown), an observation previously only noted on $CCR6^+$ and not $CCR6^-$ $CD4^+$ T cells (Monterio, et al., 2011). Strikingly, we found that permissiveness to HIV infection increases with ATRA treatment, an observation that together with elevated levels of α4β7 and CCR5 may explain the rapid HIV replication and depletion of $CD4^+$ T cells that is characteristic of acute infection in the GALT (Brenchley and Douek, 2008). ATRA has been found to suppress the differentiation of naïve $CD4^+$ T cells into Th17 T cells, and enhances their differentiation into inducible $FOXP3^+$ regulatory T cells (Johansson-Lindbom, et al., 2005; Elias, et al., 2008). Our results reflect this, showing an increase in the frequency of both Treg and $FOXP3^+$ $CD4^+$ T cells with little effect on the Th17 phenotype. The fact that FOXP3 expression and frequency is also upregulated suggest that the increase in frequency of Treg $CD4^+$ T cells is not as a result of the upregulation of activation markers. In addition the ATRA induced upregulation of Treg cells improves the suppressive function of Treg cells in acute inflammation (Menning et al., 2010). The differentiation of

naïve T cells into Treg cells by retinoic acid has the same mechanism as the retinoic acid activation of α4β7 by binding to the retinoic acid receptor-α on cells (RARα) (Hill et al., 2008). Furthermore ATRA can increase the expression of gut- homing molecules like α4β7 and CCR9 on these inducible and natural T regulatory cells (Siewert, et al., 2007, Sun, et al., 2007). Interestingly, the lamina propria of the small intestine exhibits a higher proportion of Treg cells compared to other tissues (Sun, et al., 2007). Despite retinoic acid inhibitory signals however, Th17 cells are found in the intestinal mucosa (Mucida et al., 2007). A recent study suggested that a low concentration of retinoic acid is required for Th17 cell differentiation (Uematsu, et al., 2008), but is not reflected in our data. Despite the blocking of Th17 differentiation by ATRA, α4β7 and other gut-homing receptors are still upregulated on these cells (Cha et al., 2010; Ikeda et al., 2010). It is possible therefore that retinoic acid may be required to induce gut-homing receptors but not pro-inflammatory Th17 responses. Alternatively, antigen-presenting cells that induce Th17 cells may be different from those that induce gut homing receptors in Treg cells.

As one of the mechanisms of Treg induction overlaps with that of α4β7 upregulation in the GALT, it is not surprising that we found α4β7 to be present on Treg $CD4^+$ T cells. The level of expression of α4β7 on Treg $CD4^+$ T cells however was slightly higher than on Th17 $CD4^+$ T cells, a subset previously described as mostly expressing α4β7 (Ansari, et al., 2011). Whether increased expression of the α4β7 integrin on one subset gives the population a higher propensity to home to the target tissue is unknown. Another study, suggested that $\alpha4\beta7^+$ $CD4^+$ T cells are mostly Th17 cells (Kader, et al., 2009) however this was not examined in cells under GALT conditions and our results demonstrate that a significantly larger, but still very small proportion of $\alpha4\beta7^+$ CD4 T cells are Treg $CD4^+$ T cells as compared to Th17 $CD4^+$ T cells. This reveals a significant caveat in this, and many other studies, where cells from peripheral blood are used to assess homing capabilities and functional properties of cells. Although this study does mimic the GALT environment in terms of the effect of ATRA, the ideal samples would be from gut tissue. It is interesting that $CD45RA^+$ (Naïve) Treg $CD4^+$ T cells expressed the highest levels of α4β7 in this study. Naïve Treg cell counts have been reported to be significantly reduced in HIV-infected patients, a reduction exclusively restricted to the acute infection

phase, while no difference is observed in chronically infected patients (Simonetta et al., 2012). It is possible that this subset may be important in the dissemination of HIV from the genital mucosa to the GALT as a result of its high α4β7 expression and its depletion in peripheral blood only during acute infection.

α4β7 has been described as defining a highly permissive subset of T lymphocytes (Cicala, et al., 2009) and this was confirmed in this study by sorting α4β7 populations and examining, in the absence of contaminating populations, the relative levels of p24 over 10 days. Not only this, but $\alpha4\beta7^+$ $CD4^+$T cells expressed higher levels of CCR5 than α4β7- subsets. This suggests that this integrin does offer some selective advantage to HIV infection. Moreover, a recent study in rhesus macaques has shown that the number of $\alpha4\beta7^+$ $CD4^+$ T cells at the site of transmission is a risk factor for productive HIV infection (Martinelli et al., 2013).

We also examined how permissive Th17 and Treg subsets are to HIV infection by incubating these cell populations with a T/F virus in an *in vitro* culture system, a novel comparison not present in the literature. Several studies have shown *in vitro* that Treg cells are highly susceptible to HIV infection (Oswald-Richter, et al., 2004; Chase, et al., 2008; Moreno-Fernandez, et al., 2009). Other studies have demonstrated that Th17 cells are depleted preferentially in cervical tissue and have been identified as a founder target cell subset for HIV transmission (McKinnon, et al., 2011). Evidence suggests that Th17 cells are the most prominently depleted $CD4^+$ T cell subset following HIV/SIV infection (Brenchley, et al., 2008). It is unclear in the literature whether Th17 cells are preferentially infected by virus (Kader, et al., 2009; El Hed, et al., 2010) or indirectly destroyed by bystander effects (Brenchley, et al., 2008). Our results seem to reflect the latter in that Treg $CD4^+$ T cells were substantially more permissive to acute infection by a T/F virus as compared to Th17 $CD4^+$ T cells even though these cells expressed similar levels of CCR5. Preferential infection of Th17 cells is not always found in viremic individuals (Brenchley, et al., 2008) and it has been hypothesised that the loss of Th17 cells is due to the fact that they are more susceptible to cell death caused by activation than Treg cells (Kanwar, et al., 2010). Treg and FOXP3 $CD4^+$ T cells and were not found to be

depleted as was the case with Th17 cells. However in culture, this observation may reflect the *in vivo* scenario where Treg cells have been found to increase in number following infection (Li, et al., 2011; Shaw, et al., 2011; Nilsson, et al., 2006). It has been shown that Treg suppressive capacity does not seem to be affected by HIV infection as Treg cells isolated from acutely infected HIV individuals suppress effector T cells proliferation as efficiently as Treg cells isolated from healthy donors (Kared, et al., 2008). This may be mirrored in the observation that FOXP3 expression per cell is unchanged following infection in this study as FOXP3 is essential for Treg immunosuppressive function. The rapid depletion of Th17 $CD4^+$ T cells reflects the pathology of the HIV as noted *in vivo*. Besides being highly susceptible to infection as previously mentioned, the loss of Th17 cells in the GALT environment provides a direct link between $CD4^+$ depletion and immune dysfunction. These cells function in the maintenance of the GALT mucosa, primarily through the function of IL-17 which is responsible for the strengthening of mucosal epithelial tight junctions (Blaschitz and Raffatellu, 2010). The expression of IL-17 per cell downregulated after infection may suggest a reduced function of these cells in maintenance of mucosa integrity. Despite a higher level of infectivity noted in Treg $CD4^+$ T cells as compared to Th17 $CD4^+$ T cells after 3 days, it was clear that slightly elevated levels of α4β7 in the former is not the reason for this observation as $\alpha4\beta7^+$ Treg and $\alpha4\beta7^+$ Th17 $CD4^+$ T cells showed high and similar levels of infection. Although this study uses *in vitro* culture, it mimics the *in vivo* scenario closely and may give valuable insight into the role of α4β7 in dissemination in the first few days of infection, providing details of a target for prevention of chronic HIV.

The effect of ATRA on Th17 and Treg cells as well as the role of Treg subsets in HIV has been conflicting. These conflicts exist largely as a result of the lack of a clear phenotypic profile and homogenous markers for Treg cells. Treg cells are often identified as expressing high levels of CD25 (Dieckmann et al., 2001; Jonuleit et al., 2001) which is unreliable as CD25 expression is not unique to Treg cells but is also a marker of immune activation. The most reliable and widely published phenotype, also used in this study, is a combination of CD25 high $FOXP3^+$ (a transcription factor was identified as an essential and specific factor for Treg cell development and function) and $CD127^-$ (Simonetta, et al., 2013). This phenotype is unlikely to be all encompassing as CD127 is found to be

downregulated on activated cells (Simonetta, et al., 2013) and some studies describe a distinct lack of correlation between CD25 and FOXP3 in human cells and elect to use FOXP3 alone to represent Treg cells (Suchard, et al., 2010). As a result of this we elected to use both the classical phenotypic profile and FOXP3 exclusively in our analyses to control for effects of activation on the markers.

Although many studies have examined the importance of Th17 cells in HIV as a possible founder target population of cells for HIV, as a result of their preferential depletion in genital tissue, their high expression of α4β7 (allowing for homing to the gut, a site of rapid HIV infection), high expression of CCR5 and presence in the GALT tissue (McKinnon, et al., 2011), few have examined Treg $CD4^+$ T cells as conduits for viral dissemination. We have shown evidence in this study to suggest that this may be the case and that during HIV acute infection Treg cells may be uniquely suited to fulfil this niche. Treg cells express high levels of α4β7, which not only allows migration of the cells to the GALT but binds gp120 itself (Arthos, et al., 2008). They are induced by ATRA *in vitro* as shown in this study and in the GALT (Hill, et al., 2008; Siewert, et al., 2007), a mechanism that also controls α4β7 upregulation (Iwata, et al., 2004) but did not affect Th17 expression in this study. These cells are highly permissive to HIV infection and express similar levels of CCR5 compared to Th17 cells but appeared to be expanded following infection while Th17 cells were depleted *in vitro* in this study and *in vivo* in others (Brenchley, et al., 2008; Suchard et al., 2010). Interestingly, it has been suggested that Treg cells could represent a preferential cellular reservoir of viral infection because of their apparent survival following HIV infection (Tran et al., 2008). As a result, Treg cells may be better suited to disseminating virus because they may survive long enough to do so while in contrast, Th17 cells deplete rapidly in at least 3 three days. Interestingly, the lamina propria of the small intestine and part of the GALT exhibits a higher proportion of Treg cells compared to other tissues (Sun, et al., 2007) and have been found in the genital mucosa (Jiang and Kelly, 2011), the site of sexual transmission. Therefore infection of Treg cells shortly following transmission may provide an effective mechanism of HIV dissemination by exploiting α4β7 homing to the GALT. Further exploration of this mechanism *in vivo* and in tissue explants may ultimately contribute to the exploitation of certain targets for prevention and treatment strategies.

CHAPTER 4: DISCUSSION

The role of integrin α4β7 in HIV transmission and pathogenesis is controversial. Not only can α4β7 bind gp120 (Arthos, et al., 2008) and defines a subset of cells highly permissive to HIV infection (Cicala, et al., 2009), but it may allow for viral dissemination to the GALT, the site of initial and rapid replication, by the natural homing of T lymphocytes to this region (Berlin et al., 1993; Kunkel et al., 2000). Gp120 is likely to also signal through α4β7 and LFA-1 and may increase viral transmission from cell to cell by homotypic clustering and formation of virological synapses (Arthos, et al., 2008; Birdsall, et al., 1997). Moreover antibodies directed at the V1/V2 region, epitopes of which overlap the α4β7 tripeptide binding motif, have been described as a correlate of protection for the RV144 vaccine Thai trial (Haynes, et al., 2012) and blockade of the α4β7 integrin in rhesus macaques reduced SIV viral load (Ansari, et al., 2011). A recent study however showed very little effect of α4β7 on HIV viral replication and no difference between α4β7 usage between T/F and chronic viruses using a very small sample of unmatched viruses for comparison (Parrish, et al., 2012). In this dissertation we aimed to use a larger panel of viruses from 11 individuals in the CAPRISA 002 cohort to examine the dependence on α4β7 for HIV replication and to determine the factors that may influence this reactivity. We also aimed to determine which T subsets that have been previously found to be in the GALT and genital tract express the highest levels of α4β7 which may allude to a novel route of viral dissemination from the site of transmission to the GALT.

In Chapter 2 we describe a role for α4β7 in HIV subtype C infection that changes over time and varies across individuals. In three individuals, dependence on α4β7 was higher using T/F viruses and decreased sharply during acute infection (1-3 months post-infection). This seems to reflect the *in vivo* observation that T lymphocytes downregulate α4β7 once the cells have migrated to the target tissue in the GALT (Menning et al., 2010; Wang et al., 2009b). α4β7 dependence showed an increasing trend in chronic infection over time which was slight in the first year. The spontaneous rise in endogenous IL-7 during the progression to full-blown AIDS and the consequent upregulation of α4β7 may explain the increased dependence on the integrin 3 years post infection (Cimbro, et al., 2012). We show that factors that influence α4β7 reactivity include glycan distance from α4β7-binding motif, glycan density and variable loop length in the V1/V2 region. In addition, several glycans positioned in conserved

regions of gp120 including N234, N332 and N334 showed significant differences in frequency between viruses with high and low α4β7 reactivity. Differences in frequency of glycans in the variable loops including V1/V2, V4 and V5 were also found between the two groups. This is the first study to examine the effect of position of glycans in the whole of gp160 on α4β7 reactivity. Although reduced glycosylation in V1/V2 has previously been shown to increase gp120-α4β7 binding (Nawaz, et al., 2011), and another study showed that α4β7 binding does not select for shorter less glycosylated V1/V2 loops (Etemad, et al., 2013) we observed a positive correlation between glycan density and α4β7 dependence for replication. We therefore hypothesise that it may be more pertinent as to where the glycans are and less about how many there are that affect α4β7 reactivity.

Strikingly there was an association between high dependence of α4β7 for replication at transmission and those T/F viruses that have a S/PDI/V α4β7 binding motif as well T/F viruses found in individuals diagnosed with bacterial vaginosis during acute infection. Not only this, but several cytokines in the CVL of these individuals during early infection (IL-8, IL-7 and IL-1α) positively correlated with α4β7 dependence for replication. These data suggest that the affinity for α4β7 may be determined by the transmission environment and that BV may create a bottleneck which selects for viruses that have a high α4β7 reactivity perhaps by increasing the number of $\alpha4\beta7^{+}$ $CD4^{+}$ T cells in the transmission tissue either by expression of particular cytokines or upregulation of α4β7 ligand MAdCAM-1. BV has shown to increase risk of HIV acquisition in several cohorts (Cohen, et al., 2012; Nwadioha, et al., 2011) but was only significantly associated with risk by univariant analysis in the CAPRISA 002 cohort (Mlisana, et al., 2012). This, along with the observation that T/F viruses have higher dependence on α4β7 than acute viruses, suggests that α4β7 may have a role in transmission.

In order for α4β7 to have any effect on transmission, and to disseminate virus to regions with cells highly permissive to HIV infection such as the GALT, $\alpha4\beta7^{+}$ T cells would need to be present at the site of transmission. The ligand for α4β7, MAdCAM-1 has been found to be expressed in the female genital tract and upregulated in the case of chlamydia and vaginal candidiasis which would recruit $\alpha4\beta7^{+}$ T cells to vaginal sites (Kelly et al., 2001; Wormley et al., 2001) and a number of $\alpha4\beta7^{+}$ T cells

have been described in this region including Th17 expression in the cervical tissue (McKinnon, et al., 2011). Moreover, a recent *in vivo* study using rhesus macaques showed that susceptibility to SIV was directly correlated with the number of $\alpha4\beta7^+$ T cells present in the rectum (Martinelli, et al., 2013). Before the discovery of α4β7-gp120 interaction, it was unclear how the T/F virus could bypass the unactivated cells and infect those that allow for robust productive infection, this interaction allows for a possible mechanism (Cicala, et al., 2009).

In Chapter 3 we sought to describe the subsets of $CD4^+$ T cells on which α4β7 is most prominently expressed. Treg and Th17 subsets were examined because they are both present in the GALT and genital tract and both have important roles in HIV pathogenesis. In addition ATRA is necessary for Treg induction as well as α4β7 upregulation (Sun, et al., 2007; Elias, et al., 2008; Iwata, et al., 2004) as reflected in our study, with little effect on Th17 phenotype. Treg $CD4^+$ T cells expressed slightly higher levels of α4β7 as compared to Th17 cells. In addition, Th17 cells in this and other studies were shown to rapidly deplete following HIV infection (Brenchley, et al., 2008), while Treg $CD4^+$ T cell were shown to expand (Li, et al., 2011; Shaw, et al., 2011; Nilsson, et al., 2006). Despite this, Treg $CD4^+$ T cells were significantly more permissive to infection as compared to Th17 $CD4^+$ T cells which may be as a result of Treg cells being more resistant to apoptosis (Kader, et al., 2009; El Hed, et al., 2010 Kanwar, et al., 2010) as it has been suggested that Treg cells may form part of a latent reservoir during latent infection (Tran, et al., 2008). Due to high levels of α4β7 expression and the ability to bind gp120, presence in both the genital tract and the GALT, regulation by ATRA similar to α4β7 upregulation, expansion following HIV infection and elevated permissiveness to acute infection Treg cells may be a robust vector from the genital mucosa to the GALT shortly after transmission. This subset may be better suited for this role as compared to Th17 $CD4^+$ T cells which are more susceptible to either depletion by HIV or bystander effects. There are other mechanisms of dissemination; one such example is the conserved trafficking signal in gp41 (711-714) which in SIV contributes to targeting mucosal tissues (Breed et al., 2013) but this has not been extensively studied.

Pitfalls of the study and further recommendations

While this study is the largest assessment of α4β7 dependence on HIV replication to date, only a limited number of time points within individuals were available for analysis and as such not all individuals had complete data sets. In addition, analyses involving T/F viruses were limited by the number that have been produced for other studies with several individuals not being sampled early enough to infer the T/F viruses. Future directions may include expanding the data set to limit skewing by the increased numbers of chronic time points available. In order to prove that the α4β7-binding motif may impact on α4β7 reactivity we are currently mutating the tripeptide motif sequence to assess whether higher binding can be conferred to low α4β7 binding viruses by changing the motif sequence (e.g. LDI mutated to PDI). We will assess binding capability as well as how the motif affects α4β7 dependence on virus replication. We are currently investigating the association between the P/SDI/V motif and BV diagnosis as well as cytokine expression in the CVL in the CAPRISA 002 and 004 cohort in order to make use of larger sample size. A large caveat to the study is using peripheral blood cells and treating them with ATRA to mimic the GALT environment. Multiple studies have shown that intrinsic differences exist within tissues compared to peripheral blood (McKinnon, et al., 2012; Cohen, et al., 2010) and it is possible that expression levels of homing receptor would be different in gut tissue compared to ATRA treated peripheral cells. The ideal sample tissue would therefore be gut tissue. In addition, because the infection of cells was done *in vitro* culture, an artificial situation where cells are dying and not recirculating, the ideal model for infection would be an *in vivo* model such as infecting mucosal tissue explants or rhesus macaque infection, although findings from this study mirror the *in vivo* situation. As a result of the phenotype of cells changing over time in a culture, it would be necessary to confirm the result from this study in HIV infected individuals to reduce the influence of the artifacts of culture conditions. Finally, the analysis of Treg cells as a whole has been controversial a result of the lack of a clear phenotypic profile and homogenous markers for Treg cells, but until another reliable marker is determined, this study uses the best available definition as CD25 high $FOXP3^{+}$ $CD127^{-}$ $CD4^{+}$ T cells.

Future avenues of research may include how much of role α4β7 signalling plays in cell-cell transmission and the determination of frequency of higher α4β7 binding motifs in unbiased and large population sequences. With improved studies and expansion of our sample size, it is possible that α4β7 may be a good target for the prevention of HIV transmission or reduction of the severity of inflammation.

Conclusion

In conclusion, this dissertation describes a dependence on integrin α4β7 for HIV replication which changes over time in addition to its' function as a homing receptor that is responsible for the trafficking of lymphocytes into the GALT and also serves as an attachment factor for gp120. α4β7 is not essential to establish HIV infection but the observation that α4β7 dependence changes over time and that there are factors that correlate with this change suggests that HIV obtains a selective advantage from the utilisation of the integrin. Factors that influence this interaction include glycan position, glycan density, variable loop length and P/SDV/I sequence of the α4β7-binding motif. The transmission bottleneck that seems to select for viruses that have high α4β7 reactivity may include bacterial vaginosis and elevated levels of IL-8, IL-7 and IL-1α in the genital tract. $\alpha4\beta7^{+}$ Treg $CD4^{+}$ T cells may be one of the initial populations to be infected follow HIV transmission and are well-suited to a function of viral dissemination to the GALT. This dissertation suggests that the role of α4β7 in HIV pathogenesis and perhaps more importantly transmission could be targeted to diminish the risk of HIV infection either by blockade of the integrin itself, already licensed for multiple sclerosis therapies, or by directing responses to the α4β7 binding motif in future vaccine trials.

CHAPTER 5

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CHAPTER 6

APPENDICES

Appendix A: Ethics approval



UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Miss Simone I Richardson

CLEARANCE CERTIFICATE	**M120221**
PROJECT	The Role of Integrin a4B7 Binding in HIV-1 Subtype C Pathogenesis Phenotypically Variant CD4+ Subsets
INVESTIGATORS	Miss Simone I Richardson.
DEPARTMENT	School of Pathology/Virology
DATE CONSIDERED	24/02/2012
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 24/02/2012 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Prof Lynn Morris

DECLARATION OF INVESTIGATOR(S)
To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

Appendix B: Supplementary data

Table B1: Characteristics of the HIV infection of individuals in this study

Individual	Disease progression*	BCN/nBCN†
CAP8	Rapid	BCN
CAP88	Intermediate	nBCN
CAP177	Rapid	BCN
CAP200	Rapid	nBCN
CAP206	Rapid	BCN
CAP210	Rapid	nBCN
CAP225	Intermediate	nBCN
CAP239	Intermediate	BCN
CAP244	Rapid	nBCN
CAP255	Rapid	BCN
CAP256	Rapid	BCN

*Rapid progressors were defined as having two consecutive CD4 counts < 350 cells/µl within the first 2 years of infection

† Individuals that produce broadly-cross neutralising antibodies were defined by the ability of patient sera to neutralize 40% of viruses from different subtypes

Table B2: Properties of IECs used in this study

High α4β7 dependence Low α4β7 dependence

	Time point	V1 GD	V1 length	V2 GD	V2 length	V1/V2 GD	V1/V2 length	V3 GD	V3 length	V4 GD	V4 length	C3/V4 GD	C3/V4 length	V5 GD	V5 length	gp160 GD	α4β7-binding motif	Log Viral Load *	CD4 count †	HP2/1 mAb	Act-1 mAb
8.2.00.6F	TF	4	28	1	39	5	67	1	35	4	29	7	80	1	4	30	LDI	5.32	n.d.	24.50	33.34
8.3.16.1	56 WK/ 12M	4	28	2	39	6	67	1	35	3	23	6	74	1	4	31	LDT	4.59	332	40.53	35.48
8.4.25.T25	171WK/39M	6	37	2	39	8	76	1	35	2	24	5	75	1	5	32	LDI	5.33	201	63.30	58.40
8.4.25.C10	171WK/39M	5	37	2	39	7	76	1	35	6	30	9	81	1	4	34	LDI	5.33	201	69.00	76.70
88.2.00.17-5A	TF	4	28	0	39	4	67	1	35	3	22	5	75	2	7	28	PDI	4.47	963	64.08	66.85
88.2.00.B5	5WK/1M	4	28	0	39	4	67	1	35	3	22	5	75	2	7	28	PDI	4.47	963	23.30	22.90
88.3.44.4C	30WK/ 6M	4	28	0	39	4	67	1	35	3	22	6	75	2	7	28	PDI	4.66	686	42.96	38.16
88.12.23.7A	57WK/12M	4	28	0	37	4	65	1	35	3	23	6	76	2	7	29	PDI	5	520	30.33	48.01
88.12.17.7F	57WK/12M	4	28	0	37	4	55	1	35	3	22	6	74	2	7	29	PDI	5	520	33.55	43.42
88.12.46.4B	57WK/12M	4	26	1	39	5	65	1	35	3	24	6	76	2	7	30	PDI	5	520	36.14	43.32
88.12.2F	57WK/12M	4	26	1	39	5	66	1	35	3	22	6	74	2	7	29	PDI	5	520	52.00	43.39
88.12.45.10B	57WK/12M	4	26	1	39	5	65	1	35	3	22	6	74	2	7	30	PDI	5	520	43.41	51.31
88.12.21.10D	57WK/12M	4	28	1	38	5	66	1	35	3	24	6	76	2	7	30	PDI	5	520	54.72	43.48
88.4.25.D5.4	173WK/39M	3	27	2	37	5	64	1	35	4	25	7	75	1	5	29	PDI	5.25	633	74.80	63.51
88.4.25.G3	173WK/39M	5	39	2	37	7	76	1	35	4	27	6	77	1	5	31	PDI	5.25	633	73.23	87.69
88.4.25.A3.3	173WK/39M	5	32	2	39	7	71	1	35	3	22	6	72	1	5	29	PDI	5.25	633	71.95	55.77
177.3.7AS	28WK/6M	3	18	2	39	5	57	1	35	3	20	5	72	2	7	24	PDI	5.18	444	48.38	40.87
177.3.7F.1	28WK/6M	3	18	2	42	5	60	1	35	3	20	5	71	2	5	25	PDI	5.18	444	31.03	37.07
177.12.11.3A	54WK/12M	3	26	2	42	5	62	1	35	3	17	5	69	2	7	25	PDI	4.62	370	31.77	49.81
177.12.47.6F	54WK/12M	4	25	2	42	6	67	1	35	3	20	4	71	2	5	27	PDI	4.62	370	53.32	46.52
177.12MO.1.10D	54WK/12M	5	26	2	42	7	68	1	35	3	20	5	71	2	5	28	PDI	4.62	370	44.13	48.79
177.4.25.1.11E	172WK/39M	4	27	3	42	7	69	1	35	4	23	6	75	2	7	33	PDL	4.62	293	73.45	66.43
177.4.25.28.7A	172WK/39M	6	33	3	42	9	75	1	35	3	20	5	72	2	7	33	PDI	4.62	293	63.81	65.94
200.1.06.B8A	TF	3	27	2	42	5	89	1	35	3	28	5	80	1	6	26	SDV	5.6	n.d	63.12	55.43
200.3.08.1H	21WK/5M	4	27	2	39	6	66	1	35	3	28	5	80	1	6	28	PDI	5.13	414	29.64	31.32
200.3.16.10	54WK/12M	4	27	2	39	6	66	1	35	4	28	7	80	2	6	31	PDI	4.86	367	47.45	43.37
200.3.16.13	54WK/12M	4	27	2	39	6	66	1	35	4	28	5	80	1	5	28	PDI	4.86	367	50.51	48.88
200.3.16.4	54WK/12M	4	27	2	39	6	66	1	35	4	28	7	80	1	5	30	PDI	4.86	367	53.82	48.60
200.4.25.C2.1	176WK/39M	3	24	3	39	6	63	1	35	5	28	8	80	2	6	33	PDI	5.55	193	67.83	76.62
200.4.25.E1	176WK/39M	3	24	2	39	5	63	1	35	3	23	6	75	2	8	30	PDI	5.55	193	69.84	85.57
206.1.02.B5	TF	3	25	2	40	5	65	1	35	4	25	6	77	0	7	25	SDV	5.29	n.d	50.55	48.76
206.8W.24	8WK/2M	3	25	2	40	5	65	1	35	4	25	6	77	1	7	26	SDV	5.57	365	25.23	22.99
206.6M.B4	33WK/8M	3	25	2	38	5	63	1	35	4	25	6	77	1	4	26	SDV	5.14	325	27.96	39.89
206.6M.B14	33 WK/8M	3	25	2	38	5	63	1	35	4	25	6	77	1	4	27	SDV	5.14	325	36.78	29.54
206.12M.13	56WK/ 12M	3	25	3	48	6	73	1	35	3	25	5	77	1	3	28	SDV	5.32	297	29.39	34.75
206.12M. 18	56WK/ 12M	3	25	2	37	5	62	1	35	4	25	6	77	1	4	27	SDV	5.32	297	25.39	35.96
206.39M.A4	173WK/39M	3	25	1	45	4	70	1	35	4	27	6	77	1	5	27	SDV	5.76	469	68.80	78.05
206.39M.F4	173WK/39M	3	25	1	45	4	70	1	35	3	22	5	74	1	5	26	SDV	5.76	469	61.30	70.70
206.39M.C10	173WK/39M	3	25	2	45	5	70	1	35	4	24	6	76	1	5	29	SDV	5.76	469	71.10	n.d.
206.39M.E3	173WK/39M	3	25	2	45	5	70	1	35	3	27	5	79	1	6	27	SDV	5.76	469	60.45	55.11
206.39M.E12	173WK/39M	3	25	2	45	5	70	1	35	4	28	6	80	1	3	28	SDV	5.76	469	69.80	n.d.
210.1.00.E8	TF	2	19	3	44	5	64	1	35	6	39	9	91	2	8	33	LDI	5.67	332	41.10	39.28
239.2.00.G3	TF	2	18	2	41	4	59	1	35	5	31	8	83	1	9	29	LDI	4.98	845	31.34	29.73
244.2.00.D3	8WK/2M	6	42	2	39	8	81	1	35	5	29	7	81	1	7	29	LDI	4.28	557	24.30	23.63
244.4.25.B34A	178WK/42M	3	27	2	39	5	66	1	35	5	31	7	83	1	6	29	LDI	3.73	335	61.35	63.81
244.4.25.A36A	178WK/42M	4	27	2	39	3	66	1	35	5	36	8	88	2	7	32	LDI	3.73	335	54.75	72.12
255.2.00.5	8WK/2M	3	24	3	43	6	67	1	35	4	27	7	79	1	5	29	LDI	5.29	693	15.70	22.31
255.4.25.3D	176WK/39M	7	35	3	41	10	76	1	35	5	31	7	83	1	5	34	LDI	3.95	226	74.95	82.34
255.4.25.26	176WK/39M	6	38	2	41	8	79	1	35	5	35	9	87	1	6	33	LDI	3.95	226	60.80	49.96
255.4.25.34.7D	176WK/39M	7	38	3	43	10	81	1	35	4	31	8	83	1	7	33	LDI	3.95	226	73.60	69.85
225.1.C.2.E11	TF	4	34	2	41	6	75	1	35	4	23	7	75	2	7	30	SDI	7.8	n.d	68.61	60.08
256.2.00.7C	6WK/2M	3	26	2	50	5	76	1	35	3	31	5	83	1	6	27	LDL	4.75	689	22.00	15.72
256.2.06.C7	15WK/3M	3	22	2	42	5	64	1	35	4	31	7	82	2	6	30	LDI	6.38	555	27.52	21.59
256.4.25.10	176WK/39M	3	20	3	49	6	69	1	35	5	31	8	82	1	6	31	LDL	5.01	275	65.85	75.64
256.4.25.11	176WK/39M	3	20	3	49	6	69	1	35	5	30	8	81	0	5	29	LDL	5.01	275	75.73	85.87
256.4.25.4E	176WK/39M	4	28	3	49	7	77	1	35	5	30	8	81	1	7	31	LDL	5.01	275	75.90	61.47
256.4.25.F10	176WK/39M	3	24	1	47	4	71	1	35	5	30	8	81	0	5	27	LDL	5.01	275	65.35	70.49
256.4.25.E1	176WK/39M	3	24	1	47	4	71	1	35	5	30	7	81	1	5	28	LDL	5.01	275	52.30	75.13
256.4.25.H1	176WK/39M	3	24	2	47	5	71	1	35	4	31	6	82	2	7	29	LDL	5.01	275	57.15	67.63
256.4.25.F1	176WK/39M	4	28	3	49	7	77	1	35	5	30	8	81	0	5	30	LDL	5.01	275	60.20	n.d.

GD = glycan density * Viral load measured as RNA copies/ml n.d. = not determined † CD4 count measured as cells/μl

Table B3: PCR Reaction mixture for *env* gene amplification from pcDNA3.0 vector

COMPONENT	AMOUNT (µl)
Sterile Water (Sigma-Aldrich, San Diego)	39.75
50 mM $MgSO_4$ (Invitrogen, Carlsbad)	2
20 pmol Env M primer (Rev)	0.5
TAGCCCTTCCAGTCCCCCCTTTTCTTTTA	
20 pmol Env A primer	0.5
TGCTGCTCCCAAGAACCCAA	
5 U/µl Platinum *Taq* DNA Polymerase High Fidelity	0.25
(Invitrogen, Carlsbad)	
10 x High Fidelity PCR Buffer (Invitrogen, Carlsbad)	5
600 mM Tris-SO4 (pH 8.9)	
180 mM Ammonium Sulfate	
Env DNA	1
0.2 mM dTNPs (Invitrogen, Carlsbad)[1]	1

[1] Formed by 10 µl of each of the dTNPs in 60 µl of sterile water

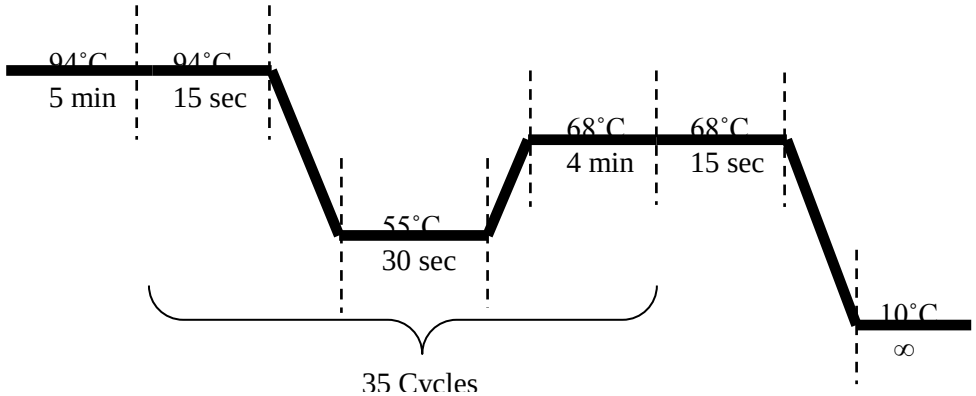
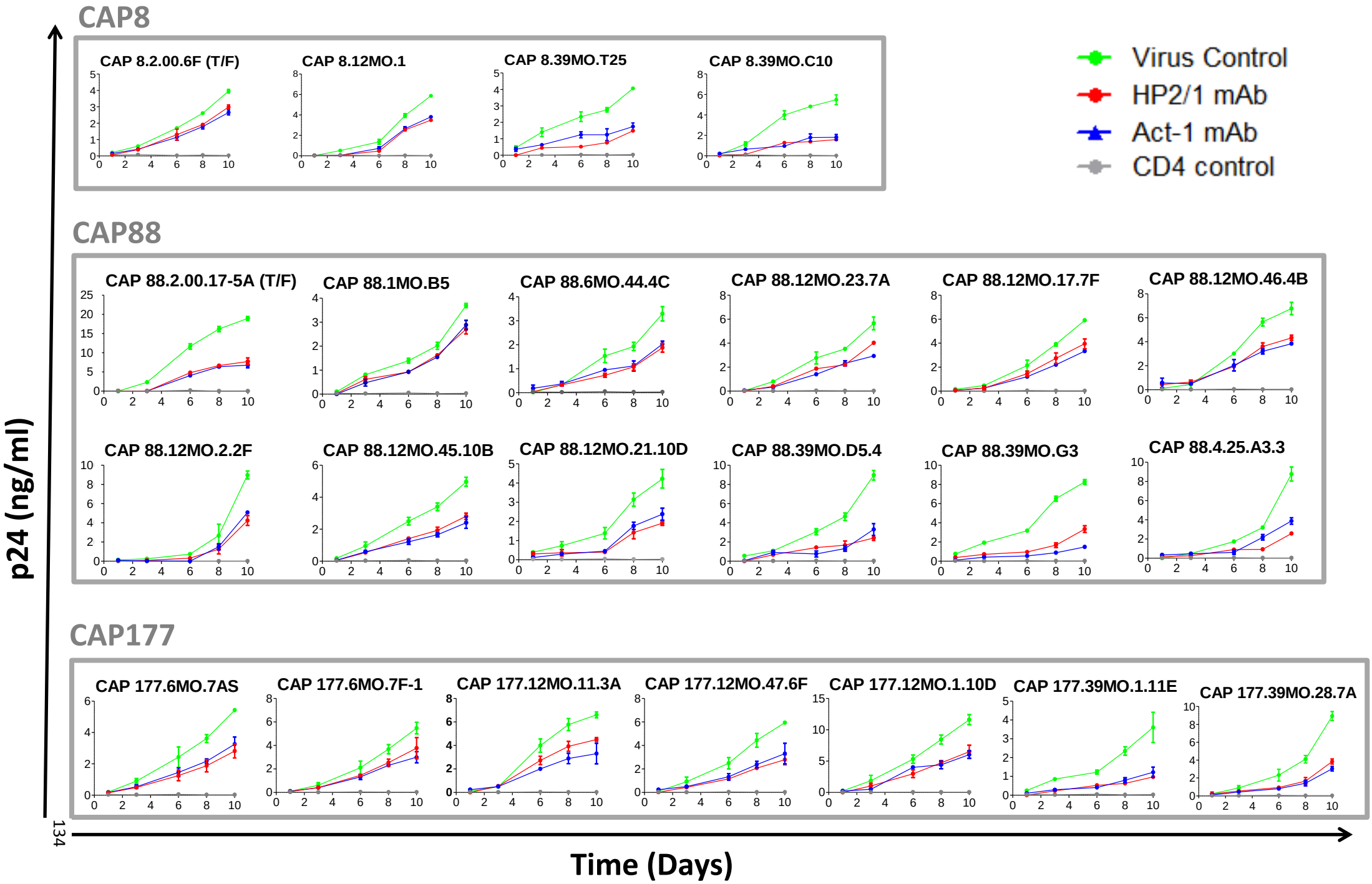
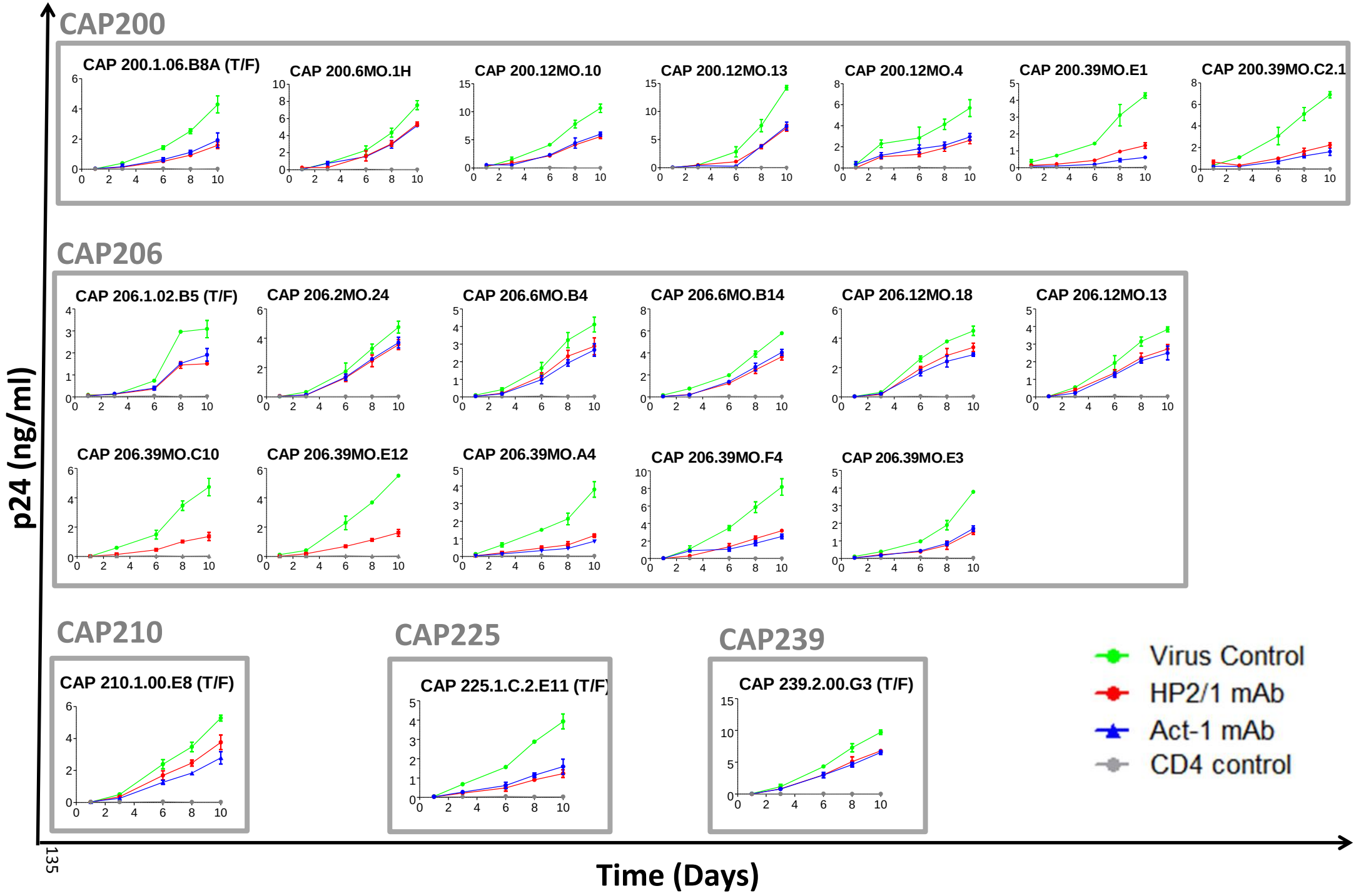


Figure B1: PCR cycle for the amplification of *env* genes from pcDNA 3.0 vector

Table B4: Reaction mixture for the digestion of pHIVΔ*env*BstEll*nef-hisD* backbone

COMPONENT	**AMOUNT (µl)**
10x Buffer D (Promega, Madison,WI)	10
10 mg/µl Acetylated BSA	1
(Promega, Madison,WI)	
10 U/µL *Bst*EII (Promega, Madison,WI)	5
Sterile water (Sigma-Aldrich, San Diego)	44
pHIVΔ*env*BstEll*nef-hisD* vector	40
TOTAL	**100**


CAP8
CAP 8.2.00.6F (T/F)
CAP 8.12MO.1
CAP 8.39MO.T25
CAP 8.39MO.C10
Virus Control
HP2/1 mAb
Act-1 mAb
CD4 control
CAP88
CAP 88.2.00.17-5A (T/F)
CAP 88.1MO.B5
CAP 88.6MO.44.4C
CAP 88.12MO.23.7A
CAP 88.12MO.17.7F
CAP 88.12MO.46.4B
CAP 88.12MO.2.2F
CAP 88.12MO.45.10B
CAP 88.12MO.21.10D
CAP 88.39MO.D5.4
CAP 88.39MO.G3
CAP 88.4.25.A3.3
CAP177
CAP 177.6MO.7AS
CAP 177.6MO.7F-1
CAP 177.12MO.11.3A
CAP 177.12MO.47.6F
CAP 177.12MO.1.10D
CAP 177.39MO.1.11E
CAP 177.39MO.28.7A
p24 (ng/ml)
Time (Days)


CAP200
CAP 200.1.06.B8A (T/F)
CAP 200.6MO.1H
CAP 200.12MO.10
CAP 200.12MO.13
CAP 200.12MO.4
CAP 200.39MO.E1
CAP 200.39MO.C2.1
CAP206
CAP 206.1.02.B5 (T/F)
CAP 206.2MO.24
CAP 206.6MO.B4
CAP 206.6MO.B14
CAP 206.12MO.18
CAP 206.12MO.13
CAP 206.39MO.C10
CAP 206.39MO.E12
CAP 206.39MO.A4
CAP 206.39MO.F4
CAP 206.39MO.E3
CAP210
CAP 210.1.00.E8 (T/F)
CAP225
CAP 225.1.C.2.E11 (T/F)
CAP239
CAP 239.2.00.G3 (T/F)
Virus Control
HP2/1 mAb
Act-1 mAb
CD4 control
p24 (ng/ml)
Time (Days)

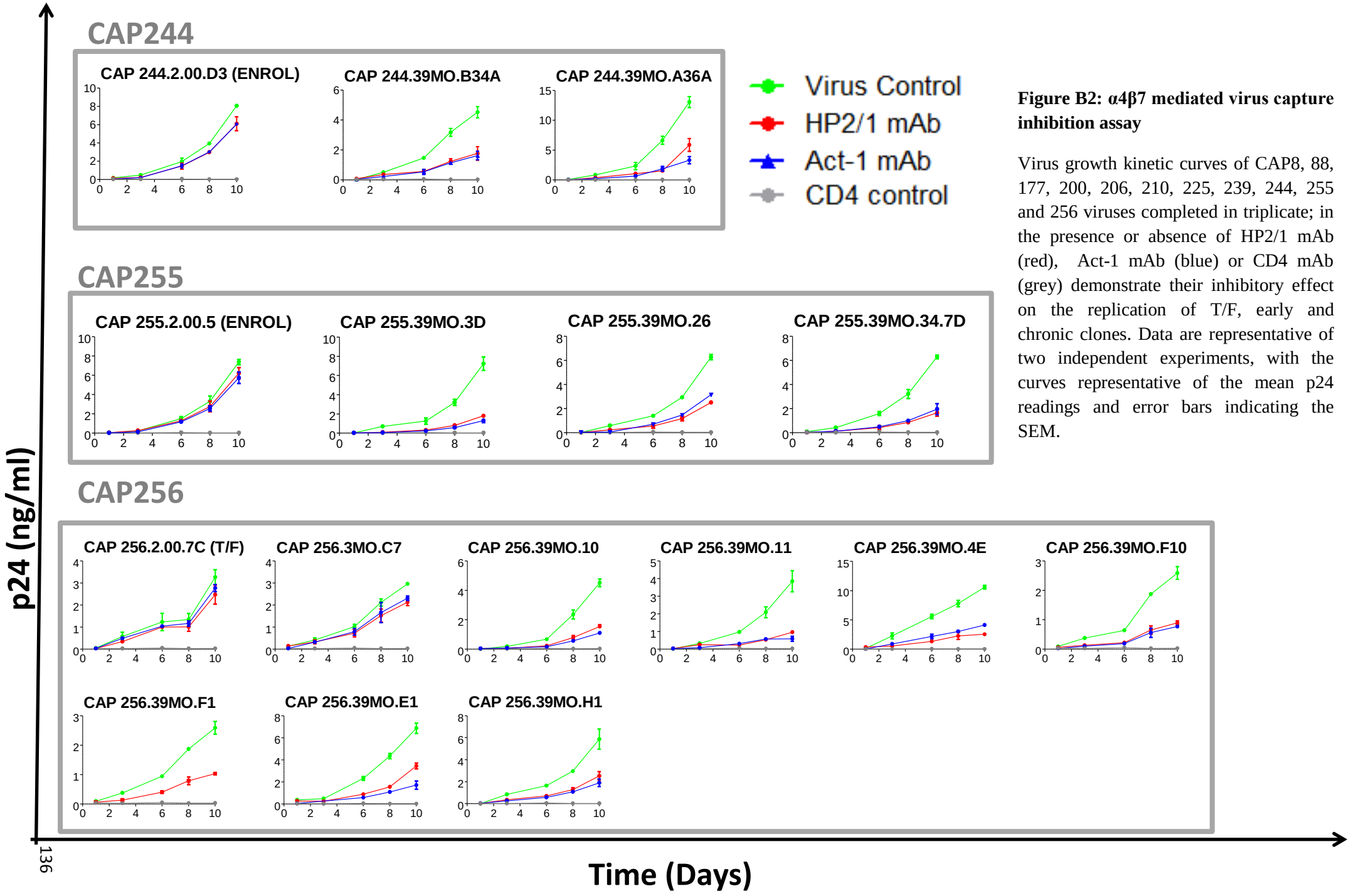


Figure B2: α4β7 mediated virus capture inhibition assay

Virus growth kinetic curves of CAP8, 88, 177, 200, 206, 210, 225, 239, 244, 255 and 256 viruses completed in triplicate; in the presence or absence of HP2/1 mAb (red), Act-1 mAb (blue) or CD4 mAb (grey) demonstrate their inhibitory effect on the replication of T/F, early and chronic clones. Data are representative of two independent experiments, with the curves representative of the mean p24 readings and error bars indicating the SEM.

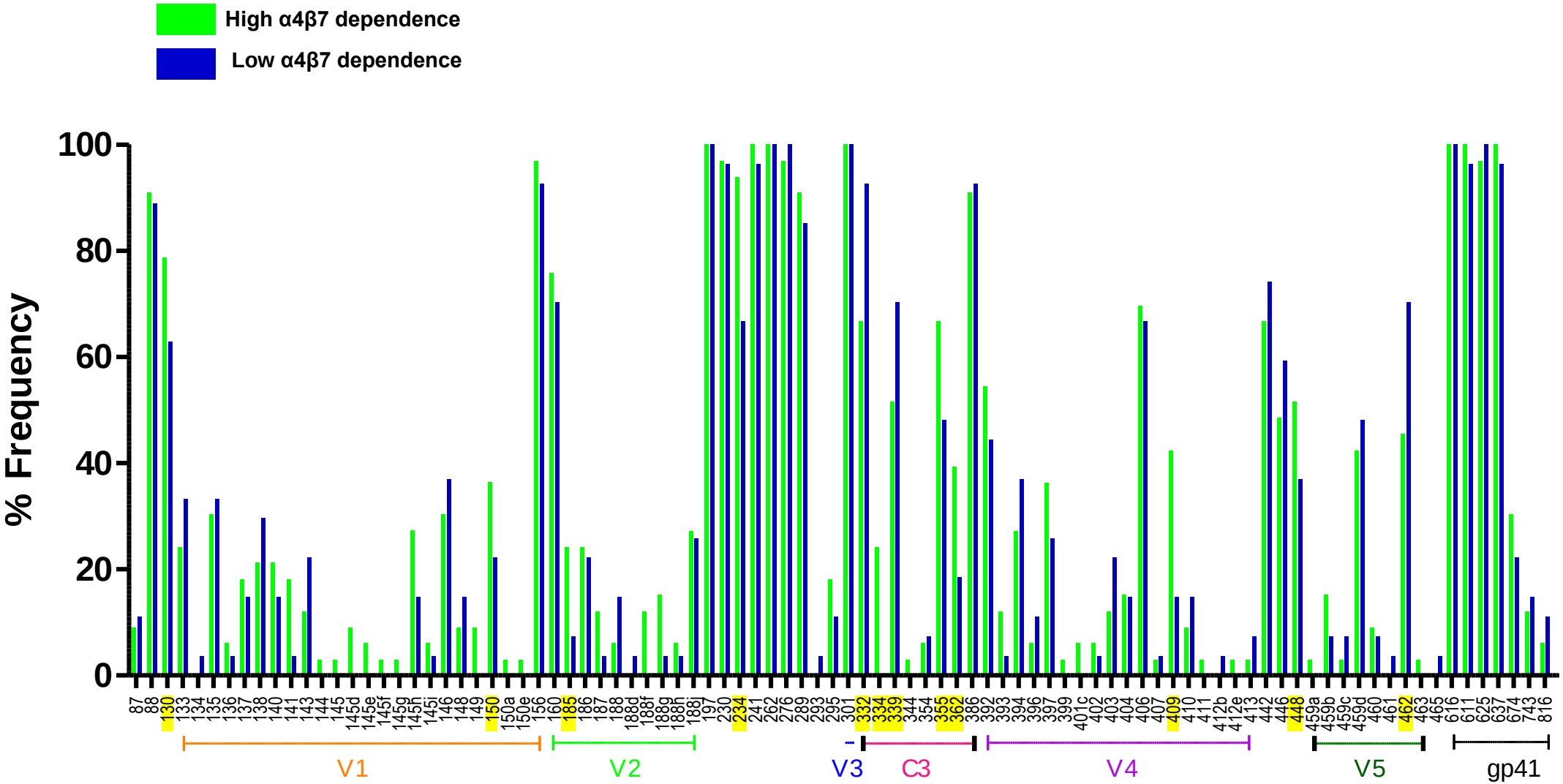


Figure B3: Glycan positions in gp160 and their frequency in viruses with high and low α4β7 dependence

The frequency of glycans at positions in gp160 across 60 viruses from 11 individuals from the α4β7 binding motif are represented as a proportion of those viruses with high and low α4β7 dependence for viral replication (shown as green and blue bars respectively). Those glycan positions where high and low α4β7 dependencies differ across at least three different individuals are highlighted in yellow. Positions of glycans within the structure are indicated below the axes.

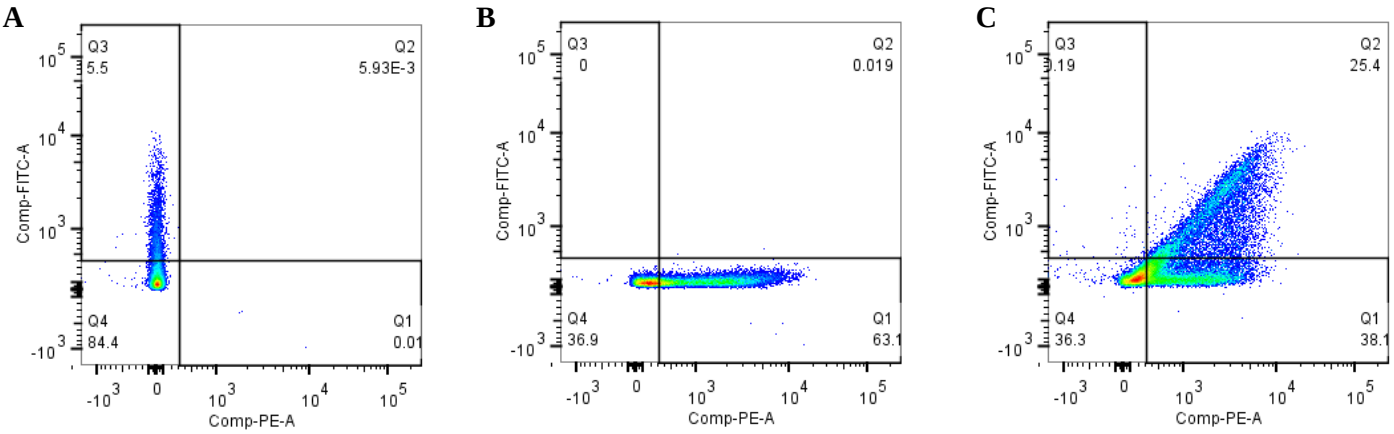


Figure B4: FMO controls for α4-PE and β7-FITC
Plots showing (A) PE FMO only stained with β7-FITC, (B) FITC FMO only stained with α4-PE and (C) α4β7 populations gated on CD4+ T cells stained with both α4-PE and β7-FITC

Feedback to applicant

Title: The role of integrin alpha4 beta7 binding in HIV-1 subtype C pathogenesis in phenotypically variant CD4 T cell subsets

Overall comments

On the whole this was a well-developed and well-presented research report on an interesting and highly relevant topic. The candidate is to be congratulated on a large body of work and on a comprehensive write-up with in depth analysis of data.

Aspects particularly well done include:

Excellent grammar and attention to detail in formatting – there were very few typographical or grammatical errors for a manuscript of this length. Manuscript is well-referenced.

The candidate demonstrates her ability to read and interpret the literature, putting together findings from diverse authors to affirm her data or commenting on areas of difference.

The candidate demonstrates experience and knowledge of two broad fields instead of only one – namely, structural biology of HIV and T cell biology. These are highly complementary fields and the merge is commendable. There was broad scope and depth of work performed for this manuscript.

There are aspects of the manuscript that I felt could have been strengthened. Particularly, there are some flow cytometry gating issues and statistical issues that are critical to clarify if publication of this work is intended. I have indicated below my feedback in 2 sections: some general comments, which may be of value for future work by the candidate but which are not expected to be changed in the current manuscript and then criticisms of the current manuscript, which should be addressed by the candidate to the satisfaction of the head of department.

A. General comments – these do not need any action by the candidate:

Style: The writing style of the candidate demonstrates that she is au fait with the literature, reads widely and can easily structure arguments. She has good tone and easily flows through descriptions of referenced findings, linking them to her work. I did, however, find the manuscript bulky and repetitive. In such a long manuscript, the repetition emphasized key points. However had the manuscript been consolidated into a shorter version, the repetition would not have been necessary. It felt like the candidate attempted purposely to flesh out the manuscript. The quality of a manuscript is independent of length and perhaps the same data could have been presented more concisely.

Study design: The topic of HIV pathogenesis is needless to say complex, and not an easy question to address. My major criticism of this study design is that there are in fact too many unknown variables to allow a conclusive answer, making the study descriptive only. By this I mean that there was not a study design where all variables were known except for the one under study. Rather there were multiple unknowns. For example: the phenotype of the founder virus in terms of alpha 4 beta 7 binding capacity

was unknown and a major question was to assess whether the founding viruses were tropic or not for the alpha4 beta7 integrin. Also the phenotype of founding cell lines infected were unknown – it is not known if these are necessarily alpha 4 beta 7 expressing or not. Then the candidate assessed whether alpha 4 beta 7 is found on Tregs and Th17 cells, but it is not known if these are founding cell types infected with HIV or not. The question would have been easier to answer if at least one of these answers were clear, ie if it was known that transmitting founder viruses are tropic for alpha 4 beta 7, it would lead one to ask if first infected cell types express the integrin, and if so, which subsets. If the first infected cells were alpha 4 beta 7 positive, it would make sense to assess the phenotype of transmitted early virus. Considering none are known – the answer of 'some viruses are alpha 4 beta 7 tropic' and "some possible responding cell types express the integrin" does not provide a strong conclusive answer of whether the integrin is necessary or permissive for early infection or for distributing early infection. Prior to embarking on the study – which potential results would have convinced the investigator that the integrin had no role in transmission?

Further, whether Th17 or Treg cells express more or less alpha 4 beta 7 integrin that other T cell subsets (Th1, Th2, NKT and non CD4 expressing cells) was not investigated. If the results of the study were to find that indeed Th17 and Tregs express alpha 4 beta 7, the inferred hypothesis that this would imply preferential HIV binding to these cell types at time of entry is an indication of pre-existing investigator bias. Other possible cell targets of early infection may also express the integrin and may be just as plausible targets as the cell types investigated.

Specific aim 2: To determine the phenotype of alpha 4 beta 7 expressing CD4 subsets.... The candidate aims to start with alpha 4 beta 7 expressing cells and look to see whether they include Tregs or Th17 cells. A different strategy could have been chosen – namely starting with Th17 or Treg subsets and investigating what proportion of each subset expresses alpha 4 beta 7. As alpha 4 beta 7 expression can be up or downregulated on multiple cell types, one could argue for and against either approach.

On page 39 and 85, the candidate mentions that donors were designated responders and non-responders to ATRA and has selected responders for further experiments. I think the candidate should be wary of describing a continuous spectrum as a binary result. This is often done for convenience, eg for selection of individuals for further experiments, but may skew interpretation. Non-responders are as interesting and physiologically relevant as responders. In fact if 25% of the group are responders, then non-responders are in fact the more predominant group and should warrant two thirds representation of a sample chosen for further experiments. The group results could also be taken as a whole for statistical analysis. In section 3.3.4 it may have been helpful to compare HIV infection in the responders versus non-responders. Why pick a responder only?

Similarly the division of viruses into high and low dependencies (eg figure 2.21) – this again has been done by definition, by defining high and low responders as those above and below the mean. This can be done with any continuous data set but does not imply any binary functional difference. Ie if boys in a class have a mean height of 1m but range from 0.8-1.2 meters, one can always discuss 2 groups – taller boys and shorter boys – even if there is no functional difference between them. They are drawn from one sample. This type of analysis may yield insights but should be done with caution.

B. Criticisms of the manuscript which should be specifically addressed by the candidate

1. Flow cytometry plots:

Figure 3.1 – parent population labelled as CD3+CD4+ live single lymphocytes but Treg plot on right hand side clearly shows CD4 positive and negative cells. CD4 should be removed from the parent block if the plots shown were not gated on CD4+ events.

Figure 3.1 Treg gating. Treg gating is controversial, as elucidated by the candidate. The chosen gating strategy for Tregs starts with CD25 plotted against CD4 and selection of the CD25hi subset. This gating strategy means that all subsequent data on Tregs is based first on where the CD25 gate is placed, which is a completely arbitrary decision at the whim of the gater. The candidate has ignored the other markers she had available to assist her with where to place the gate (such as CD127). Instead she has plotted CD25 against CD4 on the y-axis. CD4 positive cells could have (should have, according to the gating strategy described) have already been selected in the parent population , allowing plotting of CD25 against a more helpful second marker, such as FOXP3 or CD127 BEFORE the CD25 gate was set (here it is plotted against FOXP3 only after arbitrary selection of the gate, limiting the population that will appear in subsequent plots). Similarly the CD25 gate is then set a second time in an arbitrary position on the plot of FOXP3 versus CD25 (ignoring the quadrant gates) and only that smaller miscellaneous few cells is analysed for CD127 expression. The y axis of the CD127 plot is Forward Scatter – again a duplicate gate which has already been used in the parent gating strategy. The candidate is advised to regate – starting with the CD3, CD4, live single lymphocytes – then selecting the alpha 4 beta 7+ – then plotting CD25 against CD127 for enumeration of the CD25+CD127lo cells.

Figure 3.1 Why is Il-17 expression in the left hand plot labelled "Th17" clarly less than that of the lowest plot labelled "FOXP3/Il-17"? They are both plots of the same parent gate with the same x axis. It is the y axis alone (CCR6 or FOXP3) that should have different data. Are all these plots from the same sample?

Figure 3.1 flow gating of Th17 and Tregs: Legend: last line – "Il17 and FOXP3 expression alone on CD4 T cells....." should be "on alpha 4 beta 7 expressing CD4 T cells"

For publication purposes the candidate has sufficient data to answer another pertinent question – namely of the Treg and Th17 subsets, how many are alpha 4 beta 7 positive? And is this the same or different with and without HIV infection in the culture? This could be a new section and would require a new gating strategy, keeping in mind the points mentioned above. For example, Figure 3.5 middle panel - the candidate shows that ATRA treatment does not affect the Il-17 expression of the *alpha 4 beta 7 subset*. Whether ATRA treatment has increased Il17 expression by the total CD4 T cells (ie increased the proportion of CD4 T cells that are Th17s) or increased alpha 4 beta 7 expression *on the il-17 expressing cells* has not been addressed. The figure legend for figure 3.5 C should therefor say "Th17 cells defined as CCR6+Il17+CD4+**alpha4Bbeta7+"** and "Il-17+**alpha4beta7+"** werenot significantly affected..."

Figure 3.6 on page 93 and section heading 3.3.2."Treg and Th17 CD4 T cells express high levels of alpha 4 beta 7" This heading is not correct, as alpha 4 beta 7 was the parent gate. The candidate assessed what proportion of alpha 4 beta 7 expressing cells were Th17 or Treg. The figure legend "alpha 4 beta 7 is

expressed to similar levels on Th17 and Treg CD4 T cells" is also therefor incorrect as is "the grey portions represent the proportions of Treg or Th17 not expressing alpha 4 beta 7". According to gating strategy shown in Figure 3.1, I am not sure if this should be "the proportion of alpha 4 beta 7 expressing cells that are not Tregs or Th17" or the proportion of CD3CD4+ cells that are alpha 4 beta 7 negative. Similarly 'naïve Treg CD4 T cells express the highest level of alpha 4 beta 7" – this gating has not been shown. Confirm which is parent gate – the naïve Tregs or the alpha 4 beta 7 positives. Same criticism applies to page 93 last paragraph "sixty percent of Treg cells were found to express alpha 4 beta 7..."

Figure 3.7 C. Why are the two other subsets not shown? Alpha4+beta7- and alpha4-beta7+?

Figure 3.7 A. Please specify method of measurement of p24 in legend.

Figure 3.9 and text, page 97 – how was median fluorescence intensity of FOXP3 measured? On which subset and from which plot? Discuss when discuss gating strategy. Also for figure 3.5 page 92.

Figure 3.10 page 99 labels: On Page 98 candidate correctly explains her findings as a " significantly higher proportion of p24+ CD4 T cells being Treg cells as compared to Th17 cells". The corresponding figure heading however is "p24 levels in Treg and Th17 CD4 T lymphocytes" This is misleading, as is the y-axis title of figure 3.10B "% p24 CD4 T cells" and the pie charts in 3.10C and 3.10E. The y-axis title in 3.10 B should be "% **of** p24 expressing CD4 T cells" to indicate that the parent is the p24 positive population. For figure 3.10C: The total is labeled Treg as if the p24 pos and p24 negative subsets add up to 100% of the Tregs. This flow cytometric gating strategy has not been shown nor described in the text. Similarly for Th17. Representative flow plots should be shown, not only p24 on total CD4 T cells as shown in figure 3.8.

Page 101 discussion of CD45RA+ naïve Tregs – gating not shown. Not sure it adds anything to manuscript or that there are sufficient CD45RA+ and CD45RA- events to discuss.

Regarding section 3.3.4 on page 96 – assessment of permissibility of Treg and Th17 subsets to infection. These questions were addressed by comparing frequencies of Tregs and Th17 cells before and after viral infection of an unsorted culture. This however does not take into account that phenotype of the cells may change in response to the culture conditions with virus, particularly as regards *induced* Tregs. Up or downregulation of markers, apoptosis or proliferation may have occurred in response to antigenic stsimulation. Therefore this approach asks the question as to how the CD4 T cells as a group respond to culture with 2 additional variables, HIV and ATRA in the culture medium. The response observed may be due to the ATRA as well as to the HIV itself. Gating (Figure 3.8) seems to have started by choosing the p24+ cells, which presumably were then divided up into pre-set gates for Tregs and Th17 cells. An alternate gating strategy may have been preferable – namely first gating on the Tregs and Th17 cells and then asking how many of them are p24 positive. Alternatively, for Tregs at least, the cells could have been sorted first using surface markers only (by CD25+CD127lo) and then incubated with virus. ATRA treatment was an additional variable. The response without ATRA could also have been informative (as even without ATRA there is expression of alpha 4 beta 7 according to Figure 3.2).

Page 50 and 51, figure 2.7. "binding of IECs to alpha 4 beta 7 in a competition flow assay". I have two critisisms of this experiment. What is the read-out? It is not p24 that has been measured on the y axis, but % binding to alpha 4 beta 7. How was this percentage binding assessed? If by the formula on page 45 – this formula assesses the percentage binding of the blocking antibody to the integrin. So the plot is measuring the amount of alpha 4 beta 7 expressing cells remaining after blocking. How is this related to viral infection of the culture? Would virus "compete" with the blocking antibody for the integrin? If so, would it change the fluorescent staining of the integrin? Both the virus and the blocking antibody are unlabeled. So if the virus competes with the blocking antibody – what is the expected readout? **Considering that in the titration experiment, increasing amounts of blocking antibody did not correlate linearly with decreasing fluorescence of the integrin on the cells.** (fig 2.4C). Why should increasing amounts of virus therefore be expected to result in decreasing fluorescence of the integrin? If the readout depends on MFI of alpha 4 and beta 7 -**why would the gating be on the the alpha 4 beta 7 positive cells only?** This first gating step seems to be based on figure 2.5 – in which case the alpha 4 beta 7 cells are being selected **according to their MFI of alpha 4 and beta 7 BY DEFINITION**). Surely the whole population should be assessed.

Page 52 figure 2.8. This figure is a viral titration experiment, with the readout being fluorescence intensity of alpha 4 or beta 7. Would viral binding throughout the 10 day culture period necessarily affect binding of the fluorescent monoclonal on the day of staining? This would only be the case if the virus was associated with up or downregulated expression of the integrin, or interfered directly with binding of the fluorescent antibody. If virus simply uses the integrin as a transient attachment factor, then it may not affect the fluorescence intensity of the integrin per cell on the final staining day. Also – similar concerns apply regarding the gating – which if similar to figure 2.5, is limiting any change observable in fluorescence by pre-selecting (gating on) certain fluorescence intensity by definition, and excluding cells that may have shifted out of the pre-set gate.

(Similar concerns regarding the % binding to alpha 4 beta 7 would apply to all experiments using this measurement, often labelled "% dependence on alpha 4 beta 7 for replication eg figure 2.10. figure 2.11, figure 2.21, figure 2.22, figure 2.23.)

On page 39 the candidate mentions having done fluorescence minus one controls, but has not shown those flow plots. They would be good to see, perhaps in the appendix. It appears there may be issues with compensation, but it is difficult to asses without seeing the FMO data. For example – plots 2.3 last plot – diagonal "comet" shape, figure 2.5 diagonal comet shape, figure 3.3 ATRA non-reposnder diagonal comet shape (was this sample run on the same day with the same compensation matrix as the responder above?)

Was any effort made to standardize MFI's of the instrument and assay setup over time? In some sections eg figure 2.11, figure 3.2, figure 3.3 the shift in MFI is critical to the results. However it is not mentioned if all samples were run on the same day, or if they may have been run weeks apart with eg instrument services inbetween. If MFI's were compared between samples run on separate days, there should have been standardization of MFI's eg using control beads and setting gates that can be compared on various days or use of quantibrite beads or similar.

2. Statistics:

On page 46 – throughout the manuscript the candidate refers to the Mann-Whitney **t-test**. This should just be the Mann-Whitney test. Similarly the Fisher exact test is not a Fisher exact **t-test**. Similarly Wilcoxin matched pairs signed rank test on page 88

Page 50 figure 2.6 – the candidate has used a Mann Whitney test to compare before and after treatment of samples with blocking antibodies. The before and after type of analysis requires matched statistics, as the before and after groups are not two independent samples. Similarly, Kruskal Wallis would not be appropriate for figure 2.8 to compare infection of the same cells with differing concentration of virus. Further on page 89, "ATRA treatment of responders increased alpha 4 Beta 7 significantly". Incorrect choice of test. Also for figure 3.4 on page 91. Similarly on page 93 figure 3.6 pie charts – a Mann Whitney should not be used to compare two proportions of the same whole.

The same applies to figure 2.11. The viruses were from the same individual at different time points –The different time points should not be compared using a Mann Whitney test, which intended to compare medians of randomly selected sample groups.

Page 67, Figure 2.18 – 3 groups therefore candidate should use a Kruskal Wallace test, not 2 Mann Whitney tests.

For figure 3.6 on page 93 – pie charts represent the total, where the division of the pie necessarily add up to 100% of the pie. Their use with flow cytometry data can be confusing. The candidate shows two pie charts of alpha 4 beta 7 negative cells – the first which shows that Th17s comprise 50.1% of the alpha 4 beta 7 negatives, and the second which shows that Tregs comprise 60.1% of the alpha 4 beta 7 negatives. However, the two subsets should be exclusive, according to the literature. Criticisms of the gating strategy and figure legend have been addressed in comments under "flow cytometry".

Figure 3.10 B – the pale green and purple bars are proportions of the dark green and purple bars – they should not be on the same set of axes (y axis should define % of which parent)

3. General comments

Limitations of sample group: In order to assess differences between acute and chronic viruses, to try to understand the bottleneck at virus transmission, the candidate compared acute and chronic virus from the same individual. The most desirable sample group would in fact be chronic virus of the person transmitting the virus (virus "donor") to the early virus of the person receiving the virus. This is not a readily achievable sample group, although there are a few such studies, but the chronic virus studied here is in fact being used as a surrogate of the chronic virus from the virus donor. The candidate should acknowledge this limitation in the discussion. Also, on page 75 "the T/F virus was highly dependent... with a notable drop in dependence following transmission" – the T/F virus is also "after transmission" as it is a virus from the "recipient" - specify time period after transmission at which there was a notable drop.

Page 33 last sentence "establishing expression levels of the integrin on these subsets and how permissive they are to acute HIV infection may provide evidence for a route of viral dissemination from the site of transmission" – this is overstated. Just because the cells are "infectible" does not provide "evidence for a route of dissemination". It would be just as easy to hypothesize that the infectible cell types provide a route of transmission from GALT to distal sites or from lymph nodes to circulation or from genital tract to lymph nodes or that they contain virus until it is disseminated by another cell type or any other possible direction of circulation. Suggest replace with "may provide a possible route of viral dissemination from the site of transmission". Similarly regarding overstatements -I think insufficient data has been presented to draw conclusions like that on page 79 2nd paragraph "suggesting that there is a selective advantage for HIV to bind to alpha 4 beta 7" and on page 80 "suggests that alpha 4 beta 7 offers a selective advantage during transmission" . Page 79 2nd paragraph "it is possible that the bottleneck created during transmission determines how .....suggesting that there is a selective advantage for HIV to bind alpha 4 beta 7" – on what basis is this statement made?

The headings in chapter 2 methods section could be simplified. 2.2.5 "flow cytometry alpha 4 beta 7 virus binding assay" – this large heading starts with : "two forms of the binding assay were used, a competition based and a direct binding assay". The next heading is "Competition alpha 4 beta 7 binding assay" – which includes the index for % binding to alpha 4 beta 7. Then section 2.2.6 (page 45) is labelled "alpha 4 beta 7 mediated virus capture inhibition assay" - why has this been given a different name to the "competition" assay? (perhaps as it depends on subtraction of MFIs and not the "index" – in which case it should be described before the description of the "index"). Further on page 46 there is a small subheading "alpha 4 beta 7 inhibition assay" –The differences in the sections seems to relate to which virus was used and which culture conditions (ATRA, Il-2, length of culture) and the method by which p24 was measured. The difference in the experiments should be reflected in the title of each section. The readouts of most graphs in the corresponding results section seem to be the same index "% binding to alpha 4 beta 7" described on page 45 however they are labelled on the y-axis of graphs as "% dependence on alpha 4 beta 7 for replication". Also Figure 2.7 however is labelled "% binding to alpha 4 beta 7 by competition". The graph results and axes should be easy to link back to the corresponding method - ie the figure legends should correspond directly to the method descriptions, in the same order.

The difference between data included in figure 2.18 and 2.22B regarding the alpha 4 beta 7 binding motifs was not clear to me.

Page 41 section 2.2.4 until page 43 first paragraph – The second half of the paragraph under 2.2.4 mentions "for previous studies' and 2 IMCs were gifts from another laboratory– Please clarify which aspects were the candidate's work and which were previous work. Also – the TCID50 Assay – was this done by the candidate or by others? No data has been shown.

Page 52 – section 2.3.3 This section is titled "influence of alpha 4 beta 7 on HIV replication changes longitudinally over time. The data from this section are only presented in the appendix, and is difficult to absorb at a glance as there is a large amount of data (4 curves per graph, multiple timepoints per individual, different axes scales over time). Perhaps an index for % change in p24 expression, in presence

or absence of antibody (similarly to the index for alpha 4 beta 7 expression) on day 10 of culture would be an informative way to to summarize the data.

HXB2 – no introduction given to reader about consensus sequence. Should be explained. Also not on abbreviation list. First mentioned on page 21. Also page 55.

Page 57 Figure 2.12 – glycans have been highlighted in red. Are these known or potential glycans? Is there a reference or a method used by the candidate for this determination? Also on page 60 – glycan density was discussed in first paragraph. Where was glycan density data from? (reader is referred to table B2 in appendix, but I am also unclear as to where this data is from – prior experimental data from same lab or referenced data?)

Page 5 – introduction to N linked glycans. Candidate should give more introduction to the nomenclature eg difference between "N linked" and "O linked".

Page 75 "...may have a highly individual specific signature and is highly dependent on the immune system" – what is the basis for the statement regarding the immune system? Too vague

Page 79 last paragraph "our data suggest that BV creates a bottleneck that favours the transmission of viruses..." . This sentence implies causation. All data are correlative. BV may be associated with other causative factors for the bottleneck rather than the cause.

Page 88 section 3.2.4 – first half of paragraph says "viral replication was noted by intracellular p24 staining". Last half says "comparative level of p24 (ng/ml) in these cells" which infers ELISA methodology. Please clarify.

Page 90 "Tregs ...were increased in frequency .... In terms of absolute numbers" – were absolute numbers measured? If so in what unit and by what method? As a frequency of total lymphocyte count? Or of CD4 count from a diagnostic laboratory? Similar for figure 3.9.

4. <u>Unclear separation of viral and host phenotypes</u>

While the meaning of the investigator comes through, sometimes her phrasing should be clarified as host and viral "phenotypes" are often referred to collectively and not explained simply to the reader. For example:

- Abstract first paragraph: "understanding the subsets on which alpha 4 beta 7 is expressed" (this refers to host cells) "may indicate the founding phenotype for HIV infection" (is this the cellular phenotype or viral phenotype?)

- 2nd page of abstract – "Th17 cells in this and other studies were shown to rapidly deplete following HIV infection while Treg CD4 T cells were shown to expand" – should add "in vitro" or "under cell culture conditions" – otherwise sounds like the levels were measured in the patients.

- Specific aim 1 on page 34: "the role of alpha 4 beta 7 at different stages of HIV-1 pathogenesis" – this is unclear. It could refer to the role of alpha 4 beta 7 for binding viruses from different time points of infection (as performed by the candidate) or to the role of alpha 4 beta 7 expression by the host at different time points of disease progression (not performed by the candidate). Suggest narrow down to "the role of alpha 4 beta 7 binding by HIV from different stages of pathogenesis" or similar

- Chapter 2 heading " the role of alpha 4 beta 7 changes over the course of HIV subtype C infection " – this chapter focuses on virus binding and entry characteristics, not host expression of the integrin. The title could apply to either. Suggest make title more focused on virus.

- Page 37 middle paragraph "whether alpha 4 beta 7 can be defined as a transmission signature remains controversial" – a transmission "signature" should be viral. Alpha 4 beta 7 expression is host. Should rather say "alpha 4 beta 7 binding affinity/capacity"

- Discussion page 74, 2nd sentence: "the dependence on .... was associated with V1/V2 glycan density and loop length and glycans (viral factors).....and in addition CD4 count (host)..... " Followed by second last sentence of paragraph..."binding motif sequence... on the virus and dependence on the integrin for replication (virus) as well as diagnosis of bacterial vaginosis ..(host)"

- Page 76 first sentence" – The T/F viruses were significantly more dependent on alpha 4 beta 7 compared to clones from 2 months post infection, (this summarizes with the candidate's data), by which time the virus has migrated to the target tissue (this is literature regarding the host) and perhaps alpha 4 beta 7 high affinity viruses are no longer selected for (this agrees with candidate's data) or that many of these replicative competent CD4 T cells are severely and selectively diminished in number (this is literature regarding host – why would low CD4 T cells in vivo at this time-point have resulted in the experimental findings of the candidate in a controlled culture setting where there are no differences in CD4 T cell numbers?)

- Page 77 last paragraph – "increase in alpha 4 beta 7" at 3 years post infection – this is viral -add "dependence" after alpa 4 beta 7" . "interestingly, ...individuals that go on to produce BCN (this sounds like host) showed a decreased dependence for alpha 4 beta 7 at transmission" – the data on alpha 4 beta 7 dependence is from in vitro culture in healthy cells, not cells from the individual with BCN. This point should be phrased so that it is clear that it is *virus isolated from individuals who go on to produce BCN antibodies* that show a decreased dependence for alpha 4 beta 7 reactivity *in viral clones with genes isolated from transmission timepoints* compared to *viral clones* from individuals who do not produce BCN antibodies.

- Page 78 line 5 "in the first year of infection" – sounds like host. Should be "in viruses from the first year of infection"

- Page 79 last paragraph "our data suggest that BV (host) creates a bottleneck that favours transmission of viruses that have a higher dependence on alpha 4 beta 7 for replication (viral property) by either

enhancing the amount of alpha 4 beta 7 T cells in the area of transmission (host – how would this cause the experimental data in culture conditions with standard numbers of T cells? would this be a result of the viral affinity for alpha 4 beta 7?) or upregulating alpha 4 beta 7 expression on these cells (no cells from patients with bacterial vaginosis were assessed).

- Page 80 2nd paragraph: Il-1alpah, Il-8 and Il-7 (levels in CVL in host) were found to correlate significantly with the dependence of T/F viruses on alpha 4 beta 7 (in culture, this is a viral property) for viral replication in the CVL at the time of transmission (this sounds like there is data on the amount of alpha 4 beta 7 dependence by virus in CVL – misleading).

- Page 84 2nd paragraph " we sought to determine the effect of ATRA on the frequency of Th17 and Treg subsets in the peripheral blood of health donors.." – this should be "from healthy donors" (ATRA was not given to the patients)

- Page 100 first paragraph of discussion – "...cells were highly permissive to acute infection" – infection in vitro in a culture system should not be described as "acute infection"

- Page 106 discussion – "we also aimed to determine which subsets present in the GALT and genital tract express the highest levels of alpha 4 beta 7..." This was in vitro work off peripheral blood. It would be clearer to say "we also aimed to determine which subsets of T cells on newly discovered T cell subsets...." or similar.

- Page 110 conclusion – "this dissertation describes a role which changes over time for integrin..." should be for "attachment to integrin..."

- The chapter heading for chapter 3 "the role of ... subsets in early viral dissemination" is an overstatement. This chapter was in vitro stimulation work from healthy controls, albeit with viral clones from early infections. The heading could have been descriptive eg alpha 4 beta 7 expression on Treg and Th17 subtypes and permissibility for HIV infection

5. Minor errors

Abstract - bottom paragraph of first page – "Factors that influence....including N234, N332 and N334 were present more or less frequently.... Sentence too long. Remove "and" before "several glycans". In following sentence, top line of 2nd page – should read dependence **on** alpha 4 beta 7 not "dependence **of** alpha 4 beta 7 for replication at transmission"

"CVL" – first paragraph on page 2 of abstract – avoid abbreviation in abstract. CVL also not in abbreviation list and should be added

Abstract last paragraph – "which are more susceptible to either depletion by HIV or bystander effects" – "either" should come after "depletion,"

Page 3 line 5 – efforts to understand how to improve the vaccine regimen **are** ongoing, rather than "is". 2 lines later: add apostrophe in immune **system's** (not "systems")

Page 16, first line " T cells only detect pathogens though direct contact with the antigen, they must traffic to the site of pathogenic exposure" – misleading sentence. Should be "T cells detect pathogens through direct contact with MHC-presented antigen". They can therefore become activated in sites other than sites of pathogenic exposure by antigen presenting cells that have migrated there eg inductive sites. Presumably this also occurs in the inductive site.

Figure 1.8 on page 27 – not referenced.

Page 28 first paragraph " multiple sclerosis, rheumatoid arthritis, IBDs treated by blockade.." requires a reference

Page 29 Regulatory T cells – sentence beginning "FOXP3 T cells that are produced in the thymus....(Kang et al 2007) is too long and merges natural with inducible Tregs.

Page 30 Sentence starting "A significant drop in FOXP3+ Treg cells has been noted .....blood(Kinter et al 2004) Sentence is too long.

Page 32 2nd paragraph – "alpha 4 beta 7 T cells have (not has) been implicated.....Wang et al 2009b) sentence is too long.

Page 33 Rationale – abbreviation IMC is not in abbreviation list

Page 45 2nd line – p.i is not in abbreviation list

Page 52 top line – should be full stop not comma (sentence too long)

Pay attention to units. On page 48 – figure legend, 3rd last line – "pM" but graphs are "nM". On page 52 first paragraph last line – range of 0.5-25 ng – should be "ng/ul of virus".

Page 83 – low levels of CCR5 ligands – remove "ligands"

Page 91 figure 3.4 CD4 inhibition control is maroon, not grey

Comments on Simone's thesis:

Overall, the thesis is quite well put-together, has several strengths, and represents an important contribution to the HIV pathogenesis literature.

A few questions/ comments/ possible improvements:

1) The use of the term bottleneck in the Results is sometimes confusing. It is defined quite well in the intro, but then later seems to be used as 'infecting strain(s)'. Was there any impact of a4b7 dependence on the nature of the bottleneck? This is an important question, as many think infection is a stochastic process. In your work one possible conclusion might be that HIV uses a4b7 when it's available, but can still establish infection when it isn't. Of course the denominator isn't known in your case, since you are looking at cases where infection occurs, and not the many many more when it doesn't.

2) The term Mann Whitney "t test" is confusing. Also it seems that some instances where this test is used, it should probably be an actual parametric student's t test.

3) The CD4 decline analysis might need to be re-considered using repeated measures stats like a linear mixed model. This allows comparison of the rate of decline in a given person, controlling for the fact that each CD4 measure within a person will be highly correlated. Putting all of the data from multiple time points/individual in a univariate analysis is likely incorrect. For Fig2.13 this may be more appropriate since here different participants are represented in the different groups (although a Mann Whitney shouldn't be used when there are more than 2 groups). Similar comments pertain to Fig 2.11 and Fig 2.14 – the changes over time most definitely look significant, but should be subjected to repeated measures stats. This will likely be important for writing this up for publication. With respect to the CD4 decline: is a4b7 dependence result likely to result in more gut homing / more gut cd4 depletion. Is this the difference in CD4 decline a cause or effect?

4) The word data should always be plural.

5) How many of the a4b7 changes over time could be driven by neutralizing antibody escape? Are these likely to be "a4b7 escape?" The BCN correlation in fig 2.20 not well explained.

6) In a few places there are too many concepts packed into 1 paragraph, and more explanation of one particular concept /paragraph would be useful. Examples of this are on p15 P25, p65, and p68.

7) Were Mg and Ca++ used in buffers? This should insure integrins are in their activated forms.

8) The a4b7 motif itself seems de-emphasized in the analysis, even though changes in it seem to have a profound effect on a4b7 reactivity (as one might expect). It's certainly intriguing that other parts of gp160 seem important, but perhaps in a paper Fig. 2.18 should likely be more up front in the results, and then the glycan v1//v2 etc stuff.

9) Is one hypothesis that emerges from this work that HIV will use a4b7 if it's there, but that it's not required? Seems to be supported by Fig.2.22 and the BV data.

10) Is there any relation between viral fitness and a4b7 dependence?

11) The sorting approach used for the different a4b7 combinations seems best for showing differences in HIV infectivity, since many markers change over time and with stimulation/culture. What is the impact of infection/ stimulation on Th17 and Treg markers at 3 days? It seems these analysis should be approached with caution, as the conclusion that Th17 are depleted while Treg might be apoptosis-resistant – while entirely reasonable to assume – is not directly supported by the data.

12) In Fig. 3.3.2 – what are these 'other' subsets (95% of cells)?

13) For CD25, were the highest 2% gated on for each individual? If so this will not be consistent across donors, where the level of CD25 high cells should likely vary from donor to donor (with a gate set a consistent level for a set of experiments).

14) Do you think HIV drives expansion of the Treg phenotype, as has been suggested recently for T follicular helper cells?

15) How do your methods compared to Parrish, Nawaz and Etemad et al., given the differences in your gp120-a4b7 data?

16) Do Treg and Th17 levels and a4b7 expression change over the course of HIV infection in the donors where you looked at a4b7-dependence?

List of Corrections

Internal examiner
A: Flow cytometric plots

1. **Figure 3.1-parent population labelled as $CD3^+CD4^+$ live single lymphocytes but Treg plot on right hand side clearly shows positive and negative cells. CD4 should be removed from the parent block if the plots were not gated on $CD4^+$ events.**

   The flow plot referred to here does show only positive CD4+ T cells because the PBMCs used in this study were enriched for CD4 expression with a purity of 99%, as mentioned on page 38 paragraph 3. These PBMCs were gated on CD4 initially to exclude any outliers and we confirmed the purity of this singleCD4 population via various gating analyses. This gating strategy is shown on page 40 panel E.

2. **Figure 3.1 Treg gating.**
   The gating strategy that was used in this study was optimised to demonstrate the cells with the highest FOXP3 MFI, as this is currently the most specific marker for suppressive Tregs. Using CD127 instead of CD25 to set the gate is far more difficult. CD127 expression is downregulated extensively when T cells are activated, especially following HIV infection, making it difficult to discern a distinct population. As a result, most studies still choose to use the highest 2% of the $CD25^+$ population using the whole $CD4^+$ T population as a reference. The CD25 gate was not set on the $CD4^+$ $CD25^+$ plot but rather this plot was used only as a frame of reference to work out CD25 high positioning. This gate was then imposed and set on the FOXP3 $CD25^{HI}$ parameter. To avoid confusion the arrow between the CD4/CD25 plot and the CD25/FOXP3 gate has been removed on page 87 as these plots do not follow on from one another (i.e the CD25 high gate set on the CD4/CD25 plot is not the parent population for the FOXP3/CD25 plot). Rather these gates were simultaneous and the FOXP3/CD25 plot is representative of all $CD4^+CD3^+$live T cells.

3. **Figure 3.1 Why is IL-17 expression in the left hand plot labelled "Th17" clearly less than that of the lowest plot labelled "FOXP3/IL-17"?**
   This figure has been replaced with the correct image on page 87.

4. **Figure 3.1 Flow gating of Th17 and Tregs: Legend: last line**
   Many of the comments that follow on from this one appear to stem from the inaccurate representation of the gating strategy of Treg and Th17 cells in Figure 3.1. Many comments address the fact that α4β7 was the parent gate. This was only true in some cases. The gating strategy included the Treg, Th17 and FOXP3/IL-17 gated off $\alpha4\beta7^+$ $CD4^+$ T lymphocytes (now shown in the Figure 3.1 as dashed arrows) but also included $CD4^+$ T lymphocytes gated for Th17, Treg and IL-17/FOXP3 which were then assessed for α4β7 expression as illustrated by the solid lines. The previous figure only made the prior gating strategy apparent. The corrected figure is on page 87.

5. **For publication purposes the candidate has sufficient data to answer another pertinent question-namely of the Treg and Th17 subsets, how many are α4β7 positive?**
   This question has already been investigated and is on page 93 (Figure 3.6 pie chart two and three).

   And is this the same or different with or without HIV infection in the culture?
   This has been investigated (Figure 3.9 page 97) and the misunderstanding stems from the inaccuracy of communicating the gating strategy as mentioned in the previous point. The example used to illustrate this by the examiner has therefore not been addressed because

the effect of ATRA on the IL-17 expressing phenotype has been investigated and on total $CD4^+$ T cells and not on the α4β7 subset as suggested by the examiner (Figure 3.5 page 92).

6. **Figure 3.6** on page 93 and **section Heading 3.3.2…..**
   This heading and legend is correct, once again a misunderstanding evolving from the incorrect communication of the gating strategy, which has now been clarified. α4β7 was not the parent gate when determining whether α4β7 was expressed on Th17 and Treg T cells (Figure 3.6 second and third pie chart). However when determining what proportion of α4β7 expressing cells are Th17 and Treg (Figure 3.6 first pie chart), α4β7 was the parent gate. This applies to the comment on this figure's legend as well.

   Similarly naïve Treg CD4 T cells express the highest level of α4β7-this gating has not been shown. Confirm which parent gate.
   The gating strategy used to generate the data shown in Figure 3.6 B (page 93) has been added in Figure 3.1 on page 87 and it now has been specified in the legend of Figure 3.6 that the parent population naïve Treg $CD4^+$ T cells.

   The final comment for page 93 last paragraph has been addressed previously.

7. **Figure 3.7C. Why are the other two subsets not shown?**
   The other two subsets were not shown for simplicity but have now been added in on page 95

8. **Figure 3.7A: Please specify method of measurement of p24 in legend**
   Corrected on page 95 Figure 3.7 legend as being performed by in-house p24 ELISA.

9. **Figure 3.9 and text, page 97-how was median florescence intensity of FOXP3 measured? On which subset and from which plot? Also for Figure 3.5 page 92**
   Both figure legends have been corrected to say that MFIs were done on $FOXP3^+$ $CD4^+$ T cells.

10. **Figure 3.10 legend**
    As mentioned in correction 4, the gating strategy has been amended in Figure 3.1 to indicate that Th17 and Treg subsets were either the parent populations or were gated from the $\alpha4\beta7^+$population. The gating strategy was as per Figure 3.1 for Th17 and Treg subsets with $p24^+$ cells serving as the third possible parent gate (shown Figure 3.10B). In some cases where specified, $p24^+$ cells were gated from Treg or Th17 parent population (Figure 3.10 C) or from the Treg $\alpha4\beta7^+$ or Th17 $\alpha4\beta7^+$ populations (Figure 3.10 E). This explanation has been inserted on page 97 first paragraph.

11. **Page 101 discussion of $CD45RA^+$ naïve Treg-gating not shown.**
    This has been corrected as per correction number 6.

12. **Section 3.3.4 on page 96**
    The examiner expresses concern about the methodology of assessing the susceptibility of Treg and Th17 subsets to HIV infection as a result of changing phenotype following culture. While this is certainly valid, the incubation time was only 3 days and was not an extended culture. In addition this study sought to mimic the environment of the GALT as gut samples were unavailable. While the response without ATRA could have been of value, this was not the scope of the project and may be covered in work going forward. The sorting of Tregs was in the original protocol but was removed after extensive literature review revealed that the reliability of such sorts is highly questionable. In addition many Treg cells with the markers that the examiner alludes to (CD25 and CD127) express IL-17, therefore sorting using these markers wouldn't be sufficient to distinguish Tregs from Th17

cells. In addition, sorting both these populations based on surface markers is very imprecise as a result of having intracellular markers as their defining characteristics. These concerns were also addressed in the final discussion of this study.

13. **Page 50 and 51: Binding of IECs to α4β7 in a competition assay.**
The readout of this experiment is represented as "% binding to α4β7 by competition" with either virus or α4β7 inhibitory mAb. This calculation is done by the formula on page 45 as correctly determined by the examiner. As mentioned in the text in the first paragraph on page 45 "Percentage binding to α4β7 is defined as the percentage difference between the median fluorescence intensity (MFI) of PE and FITC of α4β7 transfected cells with and inhibitory mAb OR VIRUS as represented in the formula below." The formula was aimed as being an example of one of these scenarios (i.e measuring the competition between Act-1 and HP2/1 and the fluorescent antibody β7-FITC). However to make this point clear, the formula has been corrected to include "viruses".

It should be noted that the text does not state that the α4β7 inhibitory mAbs, fluorescent α4β7 mAbs AND the virus are incubated all together as alluded to by the examiner, but rather the Act-1 or HP2/1 mAbs and the fluorescent mAbs OR the virus and the fluorescent mAbs were incubated together. The HP2/1 and Act-1 mAbs were simply used as controls to illustrate that they were competing with the α4-PE and β7-FITC antibodies to a saturating degree. If virus bound to α4β7 in this assay and then was treated with fluorescently labelled α4 and β7 mAbs and compared to a control which had no virus, one would expect a decrease in MFI of α4-PE and β7-FITC relative to the control as the virus has already occupied the sites where the fluorescent mAbs would bind. These unbound fluorescent mAbs were then washed away and the readout relative to a control diminished.

Considering that in the titration experiment (Figure 2.4C), increasing amounts of blocking antibody did not correlate linearly with decreasing fluorescence of the integrin on the cells, why should increasing amounts of virus result in decreasing fluorescence of the integrin?
Figure 2.4B on page 48 shows that increasing amounts of the blocking antibodies did correlate linearly with decreasing fluorescence of the integrin, with the readout being represented as change in MFI. Figure 2.4 C to which the examiner refers, is unrelated to flow cytometry. This, as described in the legend is an alternate approach to achieve the same goal of antibody titration. While the effect was linear in the flow cytometry assay, it was not in the cellular assay. This is likely to be because of flow cytometry measuring binding capacity and the replication assay taking into account all signalling pathways. Even though the concentration is saturating for binding it is not saturating to diminish replication.

Why would the gating be on α4β7 positive cells only?
It should be remembered that the cells used for this assay were HEK293T cells which do not naturally express α4β7. Assessing the whole population is not valid as the rest of the cells do not even express the receptor of interest. Assessing the whole population does not change the result and will introduce errors because of incomplete transfection (i.e. only the α4 subunit being transfected).

14. **Page 52 Figure 2.8. Would viral binding throughout the 10 day culture period necessarily affect binding of the fluorescent monoclonal on the day of staining?**
This titration experiment, as mentioned in this chapter, was not based over a 10 day period. This was binding done with a 15 minute incubation time with the virus and an additional half an hour with fluorescent mAbs as detailed on page 45. The questions that are posed by the examiner that follow this assumption of a 10 day assay are therefore not relevant here. Any assays with a readout of "% dependence on α4β7 for replication" are indicative of an assay as detailed on page 53.

15. **Fluorescence minus one controls.**
The FMO controls have now been included as Figure B4 in the appendix on page 138. As α4β7 is an integrin and is present as a dimer, the expression pattern of the integrin on flow cytometry plots therefore appears as a diagonal as the subunits are expressed simultaneously. Although the diagonal is often a sign of poor compensation, this is how the data looks. Samples that were directly compared (HIV infected versus HIV uninfected or ATRA- and ATRA+ samples) were always run head to head. In addition fresh compensation was done with each new experiment and compensation matrixes were not reused on different days. This point has been added in Section 2.2.3 page 39.

16. **Was any effort made to standardise MFIs of the instrument and assays over time?**
A standard positive control across experiments was not used but any experiments where head to head comparisons were required were run on the same day, limiting the need to stringent standardisation. The flow cytometer is however stringently monitored for change in voltage over time by peak fluorescence beads.

B: Statistics

1. All instances of "Mann-Whitney t test" have been changed to "Mann-Whitney test" as have the Fisher exact test and Wilcoxon matched pairs sign rank test.

2. All statistics has been revisited and changed as follows:
Figure 2.6B: Repeated measures one way ANOVA with Tukey multiple comparisons post test
Figure 2.6C: Paired t test
Figure 2.7B: Paired t test
Figure 2.8A and B: Repeated measures one way ANOVA with Tukey multiple comparison post test
Figure 2.9 Wilcoxon matched pairs signed rank test
Figure 2.10 A-C Paired t tests
Figure 2.11 Repeated measures one way ANOVA with Tukey multiple comparison post test
Figure 2.13: One way ANOVA with Tukey multiple comparison post test
Figure 2.18: One way ANOVA with Tukey multiple comparison post test
Figure 2.20: One way ANOVA with Tukey multiple comparison post test
Page 89 last paragraph: Paired t test
Figure 3.4: Paired t test
Figure 3.6 A Paired t test
Figure 3.6 B Repeated measures one way ANOVA
Figure 3.7 A: Friedman's test adjusted for multiple comparisons by Dunn's post test
Figure 3.7 C: Repeated measures one way ANOVA with Tukey multiple comparisons post test
Figure 3.10 A: Repeated measures one way ANOVA, pairwise comparisons adjusted for by Tukey's method
Figure 3.10 B-F: Paired t test

3. **Figure 3.6** – concern about the gating strategy has been addressed in the previous section

4. **Figure 3.10B- the pale green and purple bars are proportions of the dark green and purple bars and shouldn't be on the same set of axes**
Both of these population subsets were gated initially on $p24^+$ $CD4^+$ T cells.

C: General comments

1. **Limitations of sample group**
   The aim of this study was to understand longitudinally what occurs in an individual with regards to α4β7 utilisation and not to access intricacies of transmission. In addition, the CAPRISA cohort does not have the donor matched pairs available and as a result this would have been an impossible study.

2. **Page 75 "The T/F virus was highly dependent…with a notable drop in dependence following transmission". Specify the time after transmission at which there was a notable drop.**
   This has been corrected as "with a notable drop 2 months post transmission" on page 75 second paragraph.

3. **Overstatements**
   The several overstatements as defined by the examiner have been corrected on page 33 last paragraph, page 79 first paragraph, page 79 second paragraph, page 79 second paragraph. All have been edited to contain the words "may, possible and may be".

4. **Headings in Chapter 2.2.5**
   The heading on page 44 has been changed to "2.2.5. Flow cytometry based α4β7-virus binding assays". In addition the competition binding assay has been labelled as A and the direct binding assay as B. These assays are both flow based assays, hence the heading. The competition assay is based on competition between virus or HP2/1 or Act-1 and the fluorescent mAbs α4-PE and β7-FITC, with binding represented as any decrease in fluorescence relative to the sample untreated with virus. The direct binding assay was done using virus that is labelled with a fluorescently labelled p24 mAb and binding there is represented as an increase in number of $p24^+$ T cells. The α4β7 mediated virus capture inhibition assay is <u>not</u> a flow based assay but is cellular in nature hence a new section title.

5. **The difference between data included in Figure 2.18 and 2.22B regarding α4β7 binding motifs was not clear to me**
   The first figure includes all viruses isolated within the first year of infection as stated in the legend and the second figure is only inclusive of T/F viruses and is also described in the legend.

6. **Page 41 section 2.24. until page 43 first paragraph for previous studies: specify which work was done by the candidate**.
   Single genome amplification and cloning of the env gene into the vector was done by others in the laboratory. The *env* genes were amplified by PCR from these in this study by the candidate. The backbone for IEC construction was gifted to the laboratory by Dr Kuritzkes but the digestion and consequent transfection was done by the candidate. The CAP210 and CAP239 IMC plasmids were gifted to the laboratory by Dr Carolyn Williamson but were transfected by the candidate. This has been made clear in the text.

   The TCID50 assays were all done by the candidate but as they were only used to titrate the input of virus to a TCID50 of 25 for assays, showing these data does not add anything to the manuscript.

7. **Page 52 Section 2.3.3. The data from this section is only present in the appendix and is difficult to absorb at a glance.**
   Data from this section is not only presented in the Appendix as suggested by the examiner but is rather summarised in Figure 2.11 and 2.12 with an index of % dependence on α4β7 for replication.

8. **HXB2 – No introduction given to reader about consensus sequence. Should be explained. Also not on abbreviation list.**

HXB2 is not a consensus sequence but rather an HIV sequence that is used to standardise numbering in sequences. This is explained on page 55 in paragraph 2.

In addition there is no abbreviation for HXB2.
This is a strain name.

9. **Page 57 Figure 2.12: Glycans have been highlighted in red. Is there a reference for a method used for its determination?**
   The determination of potential N-linked glycans was done using a tool from the Los Alamos National Database, N-Glycosite. This is detailed on page 55 in the second paragraph.

   Page 60 Glycan density is discussed in the first paragraph. Where is the glycan data from?
   How the glycan density was determined has been added in the first paragraph of page 59.

10. **Page 5- Introduction to N-linked glycans. Candidate should give more of an introduction to the nomenclature.**
    The two major types of glycosylation are now described in the first paragraph of page 6

11. **Page 77 -"…may have a highly individual specific signature and is highly dependent on the immune system" – What is the basis for the statement regarding the immune system? Too vague.**
    This sentence has been omitted.

12. **Page 79 – last paragraph " our data suggest that BV.." This sentence implies causation, but all data are correlative.**
    Changed to "BV is associated with the transmission of viruses that have..."

13. **Page 88- section 3.2.4**.- The measurement ng/ml was omitted.

14. **Absolute numbers for Figure 3.9 and Figure 3.5**
    Absolute numbers were taken as the frequency of a particular cell population represented by cell number as opposed to a percentage. This has been added on page 90 last paragraph.

D: Unclear separation of viral and host on phenotypes

1. **Abstract first paragraph:** "may indicate the founding phenotype for HIV infection" changed to " may indicate the phenotype of the first host cells infected by HIV".

2. **2nd page of the abstract paragraph 1 "**In addition, Th17 cells in this and other studies were shown to rapidly deplete following HIV infection while Treg $CD4^+$ T cell were shown to expand" was corrected to include *in vitro.*

3. **Specific aim 1 page 34:** Changed from "the role of α4β7 at different stages of HIV-1 pathogenesis" to " the dependence on α4β7 for viral replication at different stages of HIV-1 pathogenesis".

4. **Chapter 2 heading page 35:** changed from "The role of α4β7 changes over the course of HIV subtype C infection" to "The dependence on α4β7 for viral replication changes over the course of HIV subtype C infection"

5. **Page 37 middle paragraph** changed from "Whether α4β7 can be defined as a transmission signature remains controversial" to "Whether the affinity for α4β7 can be defined as a transmission signature remains controversial."

6. **Discussion page 74 2nd sentence.** There are a mixture of host and viral factors that are associated with α4β7 dependence and as a result this sentence was not edited.

7. **Page 77 2nd paragraph** "As such, the negative correlation reflects the increase in α4β7 at 3 years post infection as previously mentioned" was changed to "As such, the negative correlation reflects the increase in α4β7 dependence at 3 years post infection as previously mentioned".

   The CD4 query is addressed in the external examiners responses.

   Page 77 2nd paragraph "Interestingly, while rapid and intermediate progressors show no differences in α4β7 reactivity, individuals that go on to produce BCN antibodies show a decreased dependence for α4β7 at transmission as compared to those that do not" was changed to " Interestingly, while rapid and intermediate progressors show no differences in α4β7reactivity, viruses isolated from individuals that go on to produce BCN antibodies show a decreased dependence for α4β7 at transmission as compared to those that do not."

8. **Page 78 line 2** "Differences in the dependence on α4β7 for replication between different motifs were evident in the first year of infection" changed to "Differences in the dependence on α4β7 for replication between different motifs were evident in viruses isolated from the first year of infection."

9. **Page 79 last paragraph:** The association between BV and α4β7 dependence is likely to be as a result of host environment shaping the viral affinity. This may be because BV is increasing the expression of α4β7 on the T cells during inflammation and this provides an advantage to the viral replication of HIV and the virus adapts accordingly. This is however only a theory as BV cells were not investigated in this study as pointed out by the examiner.

10. **Page 80 paragraph 1**: "IL-1α, IL-8 and IL-7 were found to correlate significantly with the dependence of T/F viruses on α4β7 for viral replication in the CVL at the time of transmission" was changed to "IL-1α, IL-8 and IL-7 in the CVL at the time of transmission were found to correlate significantly with the dependence of T/F viruses on α4β7 for viral replication in our system."

11. **Page 84 paragraph 2**: "In this study we sought to determine the effect of all-trans retinoic acid (ATRA) on the frequency of Th17 and Treg $CD4^+$ T subsets in the peripheral blood of healthy donors in order to mimic the GALT environment where dendritic cells secrete ATRA to upregulate homing receptors (Iwata, et al., 2004)" was corrected to be "In this study we sought to determine the effect of all-trans retinoic acid (ATRA) on the frequency of Th17 and Treg $CD4^+$ T subsets in the peripheral blood from healthy donors in order to mimic the GALT environment where dendritic cells secrete ATRA to upregulate homing receptors (Iwata, et al., 2004)."

12. **Page 100 paragraph 1**: "In addition, Treg $CD4^+$ T cells express slightly more α4β7 than Th17 $CD4^+$ T cells. $\alpha4\beta7^+$ and Treg $CD4^+$ T cells were highly permissive to acute infection" was changed to "In addition, Treg $CD4^+$ T cells express slightly more α4β7 than Th17 $CD4^+$ T cells. $\alpha4\beta7^+$ and Treg $CD4^+$ T cells were highly permissive to infection by a T/F virus."

13. **Page 106 paragraph 1:** “”We also aimed to determine which subsets present in the GALT and genital tract express the highest levels of α4β7 which may allude to a novel route of viral dissemination from the site of transmission to the GALT” was changed to “We also aimed to determine which T subsets that have been previously found to be in the GALT and genital tract express the highest levels of α4β7 which may allude to a novel route of viral dissemination from the site of transmission to the GALT.”

14. **Page 110 conclusion**: “In conclusion, this dissertation describes a role which changes over time for integrin α4β7, a homing receptor that is responsible for the trafficking of lymphocytes into the GALT and also serves as an attachment factor for gp120” was changed to “In conclusion, this dissertation describes a dependence on integrin α4β7 for HIV replication which changes over time in addition to its’ function as a homing receptor that is responsible for the trafficking of lymphocytes into the GALT and also serves as an attachment factor for gp120.”

15. **Chapter heading for chapter 3** on page 82 has been changed from “The role of α4β7+ CD4+ T lymphocyte subsets in early viral dissemination” to “Expression of α4β7 on Th17 and Treg T lymphocytes and permissibility for HIV infection.”

E. Minor errors

1. **Abstract bottom paragraph of first page**
   Sentence has been shortened and in the following line “of” replaced with “on”.

2. **CVL**
   Has been added to abbreviation list and written in full in the abstract.

3. **Page 2 of abstract last paragraph**
   “This population of T cells may be more suitedfor this function than Th17 $CD4^+$ T cells which are more susceptible to either depletion by HIV or bystander effects” changed to “This population of T cells may be more suited for this function than Th17 $CD4^+$ T cells which are more susceptible to depletion either by HIV or bystander effects.”

4. **Page 3 line 5**
   “The level of efficacy was modest at 31.2% and efforts to understand how to improve the vaccine regimen is on-going” was changed to “The level of efficacy was modest at 31.2% and efforts to understand how to improve the vaccine regimen are on-going.”

   Page 3 line 8
   “immune systems” changed to “immune system’s”

5. **Page 16 first line**
   “As T cells only detect pathogens through direct contact with the antigen, they must traffic to the site of pathogenic exposure” changed to “As T cells only detect pathogens through direct contact with the MHC-presented antigen, they must traffic to the site of pathogenic exposure.”

6. **Figure 1.8 not referenced page 27**
   Adapted from Sethi, et al., 2013

7. **Page 28 1st paragraph**
   The high expression of IL-17 has been shown in multiple sclerosis, rheumatoid arthritis and IBDs, most of which are currently treated by blockade of α4β7 has been amended with a reference Tzartos, et al., 2008.

8. **Page 29 last paragraph**
   "FOXP3$^+$ T cells that are produced in the thymus from T cell progenitors are known as natural Treg cells as well as in the GALT mediated by ATRA secreted by GALT-dendritic cells in synergy with TGF-β (Sun et al., 2007) from naïve CD4$^+$ T cells known as inducible Treg cells (Kang et al., 2007) " was changed to "FOXP3$^+$ T cells that are produced in the thymus from T cell progenitors are known as natural Treg cell. However FOXP3$^+$ T cells known as inducible Tregs are also found in the GALT, the development of which is mediated by ATRA secreted by GALT-dendritic cells in synergy with TGF-β (Sun et al., 2007)."

9. **Page 30 last paragraph**
   "A significant drop in FOXP3$^+$Treg cells has been noted during SIV and HIV infected individuals (Andersson et al., 2005; Estes et al., 2006), but a number of more recent studies reported an increase in Treg T cell number following HIV infection has also been described in lymph nodes and duodenal tissue (Li et al., 2011a; Shaw et al., 2011) as well as a slight elevation in blood (Kinter et al., 2004)" was changed to "A significant drop in FOXP3$^+$Treg cells has been noted during SIV and HIV infected individuals (Andersson et al., 2005; Estes et al., 2006). However, a number of more recent studies reported an increase in Treg T cell number following HIV infection has also been described in lymph nodes and duodenal tissue (Li et al., 2011a; Shaw et al., 2011) as well as a slight elevation in blood (Kinter et al., 2004)."

10. **Page 32 2nd paragraph**
    "α4β7$^+$ T cells has been implicated in a number of therapeutic and vaccine strategies," changed to "α4β7$^+$ T cells have been implicated in a number of therapeutic and vaccine strategies"

11. **Page 33 Rationale**- IMC has been added to abbreviation list

12. **Page 45 2nd paragraph** –p.i. has been added to the abbreviation list

13. **Page 53 top line**, the line does not make sense with the suggested correction.

14. Units have been corrected on figure legend of 2.4 page 48

15. The study referred to here was referring to CCR5 ligands and not the receptor as suggested by the examiner. This has remained uncorrected.

16. The colour of the CD4 control in Figure 3.4 page 91 has been changed in the legend from grey to purple

External Examiner

1. **The examiner expresses confusion at how the term bottleneck is used in the text**. As a result several sentences have been changed to ensure that the meaning of bottleneck refers to the selection of virus strains and not the infecting strains themselves:

   "While it is not clear how this bottleneck arises apart from an increase in the number of cells expressing α4β7 attracted to the genital mucosa in response to inflammation," on page 72 last paragraph has been changed to "While it is not clear how BV may impact on the properties of viruses that establish infection, apart from an increase in the number of cells expressing α4β7 attracted to the genital mucosa in response to inflammation"

"It is possible that the bottleneck created during transmission determines how great a role α4β7 plays during this time, suggesting that there may be a selective advantage for HIV to bind to α4β7" has changed to "It is possible that the environment during transmission determines how great a role α4β7 plays during this time, suggesting that there may be a selective advantage for HIV to bind to α4β7." Page 79 paragraph 1

"Collectively, this data suggests that there is a role for α4β7 in HIV pathogenesis and that the interaction is selected for during transmission by a number of bottlenecks, one of which is the presence of BV" was changed to "Collectively, this data suggests that there is a role for α4β7 in HIV pathogenesis and that the interaction may be selected for during transmission possibly as a result of a number of factors, one of which appears to be the presence of BV." Page 81.

Was there any impact of α4β7 dependence on the nature of the bottleneck?
As the examiner points out, this study only looked at cases where successful transmission has occurred. Of the viruses that have successfully established infection, there are differences in α4β7 dependence. These differences are associated with the particular α4β7-binding motifs, the presence of BV at the time of transmission and the cytokine environment. I don't believe that the findings in this study can conclusively answer this question. The fact that T/F viruses appear to have a higher dependence on α4β7 for viral replication as compared to viruses isolated from the first year of infection suggests that α4β7 dependence may have an impact on the bottleneck. However it is also possible, and more likely in my opinion, that other correlative factors are the cause of the α4β7 dependence and not that the α4β7 dependence is the cause of the bottleneck. α4β7 dependence appears to be a selective advantage because it does allow for rapid dissemination throughout the body and α4β7 is present on many different cell types, but based on the evidence shown in this study, these outcomes are likely to be an effect of random factors. As the examiner points out, many people do believe that transmission is a stochastic process and as the body of work here suggests, α4β7 is most certainly not the determining factor for transmission to occur.

2. **The term Mann-Whitney t test is confusing.**
This was addressed in comments from the internal examiner, see "statistics". Where the term Mann-Whitney t test has been used in the text, it has been replaced with Mann-Whitney test as this test should not be referred to as a t test.

Also it seems that in some instances where this test is used, it should be an actual parametric student's t test.
All statistics have been reassessed and corrections are shown in "statistics" in the comments from the internal examiner.

3. **CD4 decline analysis and Figure 2.11 and Figure 2.14 re-analysis with repeated measure statistics**
This has been done as specified previously in the internal examiner comments under statistics.

With respect to the CD4 decline: is α4β7 dependence result likely to result in more gut homing or more gut CD4 depletion? Is this the difference in CD4 decline a cause or effect?
This correlation may also reflect that *in vivo* at time points post infection where viruses that have high α4β7 dependence are dominant systemically, CD4 T cells may increase in their capacity to home to the GALT diminishing their presence in the blood. CD4 counts undertaken in the gut may reveal a positive correlation with α4β7 dependence of systemic viruses. These data were not available for analysis. It is likely that this association is an

effect of α4β7 dependence, although clearly α4β7 dependence is not the cause of mass CD4 depletion over the course of infection. This explanation has been added on page 67 first paragraph.

4. **The word data should always be plural**
   Instances where data was not plural has been corrected.
   page 71 last paragraph
   page 77 second paragraph
   page 78 last paragraph
   page 80 paragraph 1
   page 81
   Figure B2 legend
   Abstract last page
   page 37 paragraph 1
   page 38 paragraph 2
   page 66 paragraph 1
   page 67 paragraph 1

5. **How many of the α4β7 changes over time could be driven by neutralising antibody escape? Are these likely to be "α4β7 escape?" The BCN correlation in Figure 2.20 not well explained.**
   While this an important question to answer, this is beyond the scope of this project and is likely to be the subject of further studies in our laboratory. As neutralising antibody escape is evident in the V2 region of gp120 (the region in which α4β7 is present), it is plausible that some differences in α4β7 dependence may be as a result of escape. The V2/V3 region is often referred to as being the shield for HIV's inner core and as a result is frequently the target of antibodies. This hypothesis cannot be corroborated however by the correlation as shown in Figure 2.20. This result simply shows that viruses isolated at transmission from individuals that go on to develop BCN antibodies have lower dependence on α4β7 to replicate. As a result of broadly neutralising antibodies only appearing on average two years after infection and a difference only noted at the stage of transmission, it is unlikely that this is as a result of broadly neutralisation pressure. What does cause the difference is not known as very little is known about differences between BCN and non-BCN individuals. This would need to be explored further but does suggest that BCN individuals have fundamentally different pressures at transmission that result in viruses that have a decreased dependence on α4β7 for replication. It may be important to discern these pressures so we can improve the elicitation of effective BCN antibodies in individuals. This additional point has been included in the last paragraph of page 68.

6. **In few places there are too many concepts packed into 1 paragraph**.
   The one paragraph on page 65 has been split into separate paragraphs and additional explanation has been added to the paragraph on page 68.

7. **Were Mg and Ca++ used in buffers? This should insure integrins are in their activated forms.**
   Yes, all physiological buffers used in this study contained Mg and Ca++, however were not included during the process of flow cytometric staining as they were found to interfere with staining and decrease the viability of the cells.

8. **The examiner expresses concern that the analysis with the α4β7 binding motifs is de-emphasised in the text and should be bought to the forefront in a publication.**
   The examiner's comment has been noted and in the upcoming publication, this has been rectified as were have found additional evidence for this.

9. **Is one hypothesis from this study that HIV will use α4β7 if its there but that it is not required?**
   This has been addressed in point 1 above and also added in the conclusion on page 110.

10. **Is there any relation between viral fitness and α4β7 dependence?**
    This question is a very significant one, but no assays were done in this study to adequately assess this question. This is likely to be done in future studies.

11. **The examiner expresses concern about the effect of stimulation and culture conditions when investigating Treg and Th17 HIV infection.**
    This concern has already been addressed in the internal examiner comments, however an additional statement of this pitfall has been included for clarity on page 109.

12. **In Figure 3.3.2- what are the other subsets?**
    This was not investigated as we were only concerned with Th17 and Treg subsets. It is likely there will be varying proportions of Th1 and Th2 subsets but this would have had to be done with a different staining panel as the panel used for Th17 and Treg phenotyping contained the maximum number of colours for flow cytometry.

13. **For CD25, were the highest 2% gated for each individual?**
    Yes, this was done in order to uphold the definition of what cells were CD25 high

14. **Do you think HIV drives expansion of the Treg phenotype?**
    While this has been suggested by other studies, it is also possible that Treg are simply more resistant to depletion by HIV infection. I think it also depends what tissue is being considered as completely different patterns have been noted for blood as compared to lymph, GALT or genital tissue.

15. **How do your methods compare to Parrish, Nawaz and Etemad given the differences in your data?**
    Parrish and colleagues did not take signalling of the integrin into consideration and did not titrate the inhibitory mAb Act-1, but rather worked with what was a saturating concentration used in binding assays. In addition, they used unmatched IMCs as compared to matched longitudinal IECs, a different virus expression system. Nawaz used a very limited pairing of viruses (3) to investigate the role of glycans and these were mutated to exclude glycans while this study uses 60 viruses occuring in natural HIV infection. In the pair of viruses that are common to both studies, the same dependences were observed (CAP 88 T/F and 12m viruses) with T/F having a higher binding to α4β7 as compared to the 12 month virus. This, despite the fact that the Nawaz study used monomeric gp120 while our study used replicative virus. Contrary to observations by Nawaz and colleagues, the effect of α4β7 on replication has been deemed to remain unaffected by shorter glycosylated V1/V2 loops in matched early (1-6 months) and chronic (ranging between 24-47 months) viruses from eight individuals. Although intriguing, these data from Etemad and colleagues generalise early viral time points, do not consider T/F viruses and defined chronic viruses at different time points across individuals and are only subtype A where as this study is subtype C only.

16. **Do Treg and Th17 levels and α4β7 expression change over the course of HIV infection in the donors where you looked at α4β7 dependence?**
    This has not been assessed in this study, as only healthy donors were assessed for Th17 and Treg expresssion and then assessed following HIV infection *in vitro*. This would be a very interesting question and is planned for further study.

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CERTIFICATE OF SUBMISSION TO BE SIGNED BY ALL SUPERVISORS OF HIGHER DEGREE CANDIDATES

SIMONE IRENE RICHARDSON (Name) | 3142071 (Student Number) | 0823836563 (Telephone) | simone@nicd.ac.za (E-mail)

Candidate for the degree of MSC (MED) VIROLOGY (degree) has submitted ~~his/her~~ thesis/dissertation/~~research~~ report

Entitled:

THE ROLE OF INTEGRIN $\alpha_4\beta_7$ BINDING IN HIV-1 SUBTYPE C PATHOGENESIS IN PHENOTYPICALLY VARIANT CD_4^+ T CELL SUBSETS

1. Has this ~~thesis~~/dissertation/~~research report~~ been submitted with the acquiescence of the supervisor?

Yes	No
✓	

2. To the best of your knowledge are you able to verify that:

		Yes	No
2.1	this is the candidate's work except as otherwise stated by the candidate?	✓	
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2.3	the candidate has acknowledged wherever any information used in the thesis, dissertation or other work has been obtained by him/her while employed by, or working under the aegis of, any person or organization other than the University or its associated institutions?	✓	

I certify that this ~~thesis~~/dissertation/~~research~~ report has the approval of the Animal Ethics Committee/ Committee for Research on Human Subjects and the Number of the Certificate of Approval is: M120221

Name of Supervisor:	PROF LYNN MORRIS	Telephone:	(011) 386-6332
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Date:	17 February 2014		
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14. Name of supervisor: PROF LYNN MORRIS

Discipline VIROLOGY

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Name of second supervisor (if more than one): DR NONHLANHLA MKHIZE

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13 February 2014

Postgraduate Office
Faculty of Health Sciences
University of the Witwatersrand
Johannesburg

Award of Degree MSc (Med): Ms Simone Richardson

Thesis Title: The role of integrin $\alpha_4\beta_7$ binding in HIV-1 subtype C pathogenesis in phenotypically variant CD4+ T cells

Ms Richardson has been awarded her MSc (Med) subject to her supervisors' approval. We have reviewed the examiners report and the corrected thesis of Ms Richardson and we are satisfied that all the corrections and suggestions made by the examiners have been satisfactory and adequately addressed.

Yours Sincerely,

Prof. Lynn Morris
Head: HIV Virology Laboratory

Dr. Nono Mkhize
Supervisor

Chairperson: Prof Algondia Perez Deputy Chairperson: Dr Mohamed Randera CEO: Sagie Pillay
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Faculty of Health Sciences

University of the Witwatersrand

Dear Sir/Madam

PhD Student : Ms Simone Richardson

Research topic : The role of integrin α4β7 binding in HIV-1 subtype C pathogenesis in phenotypically variant CD4+T cells

This is to confirm that Ms Richardson has made corrections to her MSc thesis as recommended by the examiners to the satisfaction of his supervisor, Dr Mkhize and head of the HIV laboratory, Prof Morris. Ms Richardson has met all the requirements for the degree of MSc and therefore can be awarded the degree as per recommendation of the Faculty of Health Sciences Postgraduate Committee.

Yours sincerely

Professor J.N. Mahlangu
Head of School of Pathology