Imperial College London

Antibody Effector Functions Critical to Mucosal Protection Against HIV-1

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Declaration of Originality

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Abstract

Human Immunodeficiency Virus-1 (HIV-1) is a retrovirus that was identified more than thirty years ago as the cause of Acquired Immunodeficiency Syndrome (AIDS). The only HIV-1 vaccine trial to demonstrate any potential, the Thai RV144 HIV-1 vaccine trial, was correlated with antibody-dependent cellular cytotoxicity (ADCC) mediated by non-neutralising antibodies (nnAb). This has led to speculation that ADCC and other antibody-dependent cellular effector functions might provide an important defence against mucosal acquisition of HIV-1 infection. However, the ability of antibody-dependent cellular effector mechanisms to impact on early mucosal transmission events is unknown and will depend on a variety of parameters including effector cell type, frequency, the class of Fc receptor (FcR) expressed, the number of FcR per cell and the glycosylation pattern of the induced antibodies. This project seeks to test the hypothesis that the protective effect of HIV-1 antibodies is likely dependent upon their functional activity in mucosal tissues, which may not be predicted by conventional neutralisation assays.

To test this hypothesis, initial work was undertaken to characterise and compare the frequency and phenotype of IgG (CD16 [FcγRIII], CD32 [FcγRII] and CD64 [FcγRI]) and IgA (CD89 [FcαR]) receptor expression on effector cells within male and female genital mucosal tissue, colorectal tissue and whole blood. The frequency of FcR expression on CD14+ monocytic cells, myeloid dendritic cells and natural killer cells were similar across the three mucosal tissue compartments, but significantly lower when compared to the FcR expression profile of effector cells isolated from whole blood, with many cells negative for all FcRs.
Subsequently, the activity of neutralising (nAb) and nnAb within the mucosal explant inhibition assay with penile glans, ectocervical and colorectal tissue was investigated. The results showed that, within the three mucosal tissues, none of the nnAb tested were efficient at preventing infection. Conversely, nAb demonstrated good levels of inhibition in all three mucosal tissue types.

Finally, to determine whether a potential influx of effector cells from the periphery could impact on antiviral activity, the ability of nAb and nnAb to reduce HIV-1 infection in the presence of whole blood were assessed. Furthermore, the binding of these antibodies to HIV-1 infected cells was investigated. The data showed that nnAb were unable to reduce infection of CD4+ cells, although they showed some ability to reduce onward viral transmission. However, nAb were able to reduce the level of infection in this assay system. Antibody binding to infected cells did not correlate with reduction in HIV-1 infection.

In summary, the combined data demonstrated that nnAb were insufficient at preventing HIV-1 transmission. Conversely, nAb were capable of reducing HIV-1 infection in all of the assay systems investigated, indicating that it is nAb that should be the primary focus of future vaccine discovery.

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Abbreviations

Ab	Antibody
Ad	Adenovirus
ADCC	Antibody Dependent Cellular Cytotoxicity
ADCP	Antibody Dependent Cellular Phagocytosis
ADCVI	Antibody Dependent Cellular Viral Inhibition
AIDS	Acquired Immunodeficiency Syndrome
AMP	Antibody-Mediated Protection
APC	Antigen Presenting Cell
ART	Anti-retroviral Therapy
AZT	Azidothymidine
BCR	B cell Receptor
bnAb	Broadly Neutralising Antibody
CCR	C-C Chemokine Receptor
CD	Cluster of Differentiation
CDC	Centre for Disease Control
CRF	Circulating Recombinant Strains
CXCR	C-X-C Chemokine Receptor
DC	Dendritic Cell
DH	Dispase High
dIg	Dimeric Immunoglobulin
DL	Dispase Low
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-linked Immunosorbent Assay
Fab	Fragment Antigen Binding
FACS	Fluorescence-Activated Cell Sorting
Fc	Fragment Crystallisable
FcR	Fc Receptor
FDA	Food and Drug Administration
FMO	Fluorescence Minus One
g	Centrifugal force of acceleration due to gravity

GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
h	Hour
HAART	Highly-Active Antiretroviral Therapy
HCDR	Heavy-chain Complementarity Determining Region
HIV-1	Human Immunodeficiency Virus – 1
HIV-IG	Human Immunodeficiency Virus Immunoglobulin
HLA	Human Leucocyte Antigen
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LPS	Lipopolysaccharide
ITAM	Immunoreceptor Tyrosine-based Activation Motif
ITIM	Immunoreceptor Tyrosine-based Inhibition Motif
LC	Langerhans' Cell
mAb	Monoclonal Antibody
mDC	Myeloid Dendritic Cell
MFI	Mean Fluorescent Intensity
MHC	Major Histocompatibility Complex
mIg	Monomeric Immunoglobulin
min	Minutes
MIP	Macrophage Inflammatory Protein
MPER	Membrane-Proximal External Region
MTCT	Mother-to-Child Transmission
nAb	Neutralising Antibody
NK	Natural Killer Cell
NKT	Natural Killer T cell
nnAb	Non-Neutralising Antibody
pAb	Polyclonal Antibody
PAMP	Pathogen-Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline plus Tween
PHA-L	Phytohaemagglutinin-L

RBC	Red Blood Cells
RNA	Ribonucleic Acid
RT	Room Temperature
SIV	Simian Immunodeficiency Virus
SHIV	Simian/Human Immunodeficiency Virus
SHM	Somatic Hypermutation
STI	Sexually Transmitted Infection
TCR	T cell Receptor
TH	Thermolysin High
TL	Thermolysin Low
TLR	Toll-Like Receptor
TM	Thermolysin Medium
TNF	Tumour-Necrosis Factor
URF	Unique Recombinant Strain
WHO	World Health Organisation

CHAPTER 1 – INTRODUCTION

1 Introduction

1.1 Introduction to HIV and AIDS

1.1.1 The HIV Pandemic

Human Immunodeficiency Virus (HIV) is a single-stranded RNA virus of the retroviridae family, initially described as the causative agent of Acquired Immunodeficiency Syndrome (AIDS) in the early 1980s [1, 2]. Since then, HIV has become a worldwide pandemic, infecting over 70 million people in total, with 36.7 million [34 million – 39.8 million] people living with the infection in 2015 (www.unaids.org).

1.1.2 Origin and Classification of HIV

HIV is a zoonotic disease originating from Simian Immunodeficiency Virus (SIV), a virus that infects non-human primates. HIV can be subdivided into HIV-1 and HIV-2, with HIV-1 originating from chimpanzees and gorillas and HIV-2 originating from sooty mangabeys [3]. Genetic analysis of HIV-1 has shown that SIV has crossed the species barrier from chimpanzees (SIV_{cpz}) and gorillas (SIV_{gor}) on at least four distinct occasions, leading to the establishment of groups M, N, O (SIV_{cpz}) and P (SIV_{gor}). Of these four groups, N, O and P have caused only a small number of HIV-1 cases in humans, whereas group M is responsible for the global HIV-1 pandemic [4-6]. Group M originated in chimpanzees (*Pan troglodytes troglodytes*) in Cameroon [7] and crossed over to humans in the late 19^{th}/early 20^{th} Century in the Kinshasa region of the Democratic Republic of Congo (DRC) [8, 9]. Group M can be further subdivided into clades A-K and

circulating (CRF) and unique recombinant strains (URF), of which clade C is the predominant HIV-1 strain found worldwide (Figure 1.1) [10].

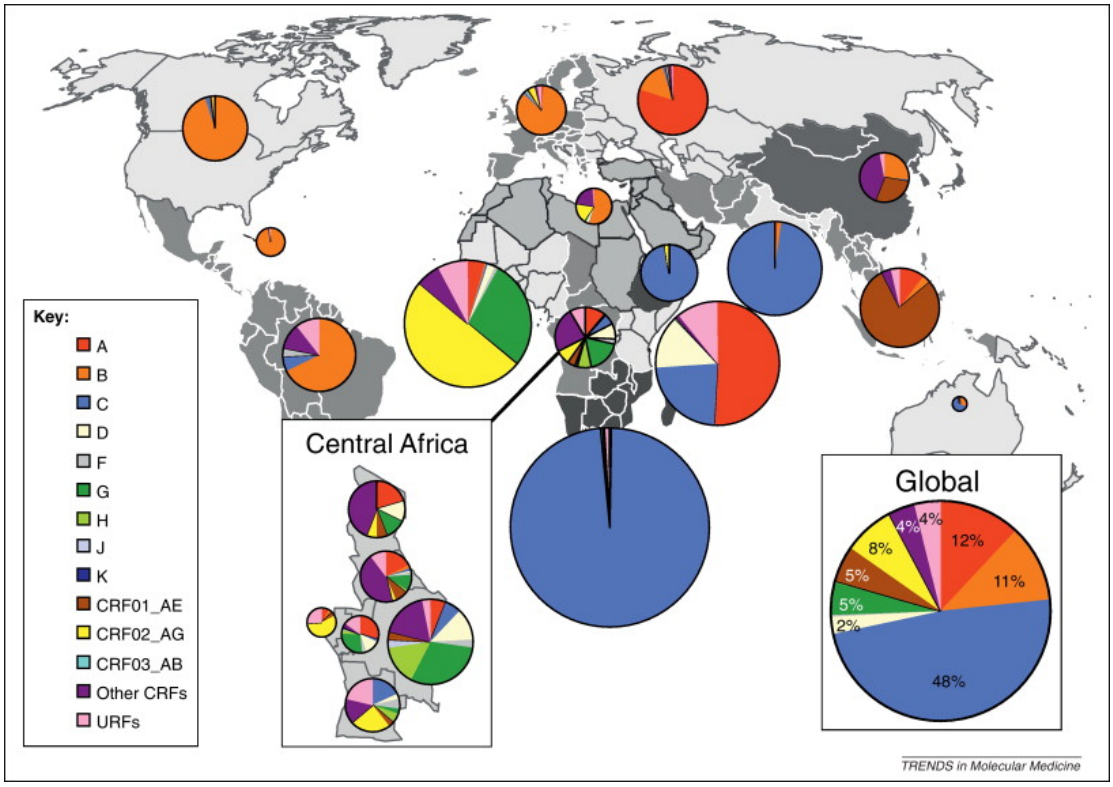


Figure 1.1 Global distribution of HIV-1 subtypes and recombinants from 2004-2007.
Pie charts are superimposed over regions to represent the distribution of HIV-1 subtypes and recombinants. The size of the pie charts corresponds to the number of people living with HIV-1 within that region. *Diagram taken, with permission, from J. Hemelaar (2012) Trends in Molecular Medicine* [11].

1.1.3 HIV-1 Clinical Symptoms

1.1.3.1 Acute Infection

During the first two weeks of HIV-1 infection, there are few symptoms. After this point, the symptoms reflect those seen for other viruses including high fever, nausea, headache, fatigue and a rash, although the exact symptoms vary between viral clade. This represents the stage at which the virus has already

rapidly multiplied within the body, leading to a 'cytokine storm' and clinical symptoms. Once the initial symptoms resolve, a viral set-point is reached whereby there is a period of relative calm, which can last for several years [12].

1.1.3.2 Progression to AIDS

AIDS is classified as a syndrome and not a disease itself, as there is no one set of symptoms that define AIDS. As the HIV-1 infection progresses, a gradual decline of CD4+ cells leads to immunodeficiency and susceptibility to a range of opportunistic infections. The Center for Disease Control and Prevention (CDC) and World Health Organisation (WHO) have produced a comprehensive list of these infections and diseases, including, but not restricted to Kaposi's sarcoma, Candidiasis and Cryptosporidiosis. Once an HIV-1 infected individual presents with a CD4+ T cell count of <200 cells/μL blood and one of the AIDS-defining illnesses listed by the CDC/WHO, they are classified as having AIDS (https://www.aidsetc.org/guide/hiv-classification-cdc-and-who-staging-systems). However, improvements in HIV-1 therapy mean it is not the death-sentence it once was. With treatment, the symptoms can be reversed, leading to individuals being re-classified as AIDS-free, a status they can maintain for many years, providing there is correct adherence to the HIV-1 medication.

1.1.4 The HIV-1 Genome

HIV-1 has a 9.7kb genome comprising numerous genes that function to form the individual components of the virus [13]. Proteins critical to the viral structure are encoded by *gag, pol* and *env* genes, while proteins essential for

viral regulation are encoded by *tat* and *rev* and lastly, accessory regulatory proteins are encoded by *nef, vpr, vif* and *vpu* (Figure 1.2).

1.1.4.1 Env

The *env* gene encodes for the viral envelope protein, which forms the envelope spikes that enable HIV-1 to attach and enter the CD4+ target cells. The envelope spikes are formed of a gp160 heterodimer, which is made up of two non-covalently bound subunits, gp120 and gp41 [14, 15].

1.1.4.2 Gag

The *gag* gene encodes for the proteins that make up the internal viral core, which houses HIV-1 RNA and replication machinery. These proteins include p17, p24, p7, p6 and two spacer proteins (SP1 and SP2) [16, 17].

1.1.4.3 Pol

The *pol* gene encodes for HIV-1 enzymes involved in replication including reverse transcriptase and integrase, as well as protease, an enzyme involved in the final stages of HIV-1 maturation. Reverse transcriptase is a viral enzyme that converts viral RNA into viral DNA to allow for integration into the host DNA. This integration is aided by the viral enzyme integrase. Post-replication, the virions mature with the help of viral proteases, which cleave the newly formed Gag and Gag-Pol proteins [18, 19].

1.1.4.4 Rev

The *rev* gene encodes for the Rev protein, which is critical to the regulation of HIV-1 protein expression. Without Rev, HIV-1 structural genes are incapable of translocation out of the nucleus, hindering the viral replication cycle [20].

1.1.4.5 Tat

The *tat* gene encodes for the trans-activator of transcription (Tat) protein, promoting transcription of HIV-1 RNA species from integrated DNA by binding to the trans-activation response (TAR) element and recruiting transcription factors needed to elongate the transcript from the long terminal repeat (LTR). Furthermore, Tat can be found outside virally infected cells in the circulation of HIV-1 infected individuals. Here, it is taken up by uninfected CD4+ T cells, leading to their death and thus increasing the progression towards AIDS [21, 22].

1.1.4.6 Nef

The *nef* gene encodes for negative regulatory factor (Nef), which is a small protein that acts to increase the virulence of HIV-1 by maintaining T cells in an activated state and lowering the threshold for CD4+ cell activation. HIV-1 is fully functional without *Nef*, although loss of the gene impacts on viral virulence [23].

1.1.4.7 Vif

The *vif* gene encodes for the viral infectivity factor (Vif) protein, which serves to interrupt the antiviral activity of the human enzyme apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G (APOBEC3G). The role of APOBEC3G is to interfere with the replication process of retroviruses by mutating viral nucleic acids. Without *Vif*, APOBEC3G leads to the development of non-infectious virions [24-26].

1.1.4.8 Vpr

The *vpr* gene encodes for viral protein r (Vpr), which is involved in the regulation of nuclear import of the HIV-1 pre-integration complex (PIC).

Furthermore, it is necessary for enabling viral replication in non-dividing cells, such as macrophages [27].

1.1.4.9 Vpu

The *vpu* gene encodes viral protein unique (Vpu), which functions to enhance virion release from virally infected cells by counteracting tetherin. Furthermore, Vpu enhances degradation of the CD4 receptor within the endoplasmic reticulum of infected cells. This leads to the characteristic downregulation observed in productively infected HIV-1+ cells. Downregulation of CD4 prevents binding of Env from newly formed HIV-1 virions to CD4 within the endoplasmic reticulum and enhances the assembly of HIV-1 virions [28, 29].

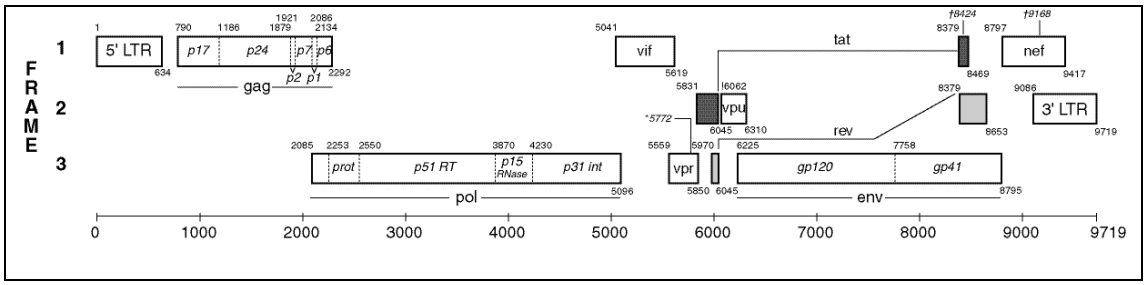


Figure 1.2 HIV-1 viral genome.

Diagram from http://www.hiv.lanl.gov/content/sequence/HIV/MAP/landmark.html

1.1.5 The Structure of HIV-1

HIV-1 is an enveloped, positive-sense virus containing two copies of single-stranded viral RNA, which is spherical in structure. From the envelope, gp160 proteins protrude, which are used for attachment and entry into target cells. Compared to other viruses, HIV-1 envelope proteins are relatively few in number, with each virion containing an average of 14 spikes [30].

Inside the envelope, matrix proteins (p17) form the inner viral protein shell, which confers structural support to the virion. The capsid is comprised of approximately 250 capsid proteins (p24), which form a stable fullerene cone and also contains the RNA and proteins involved in replication [31] (Figure 1.3).

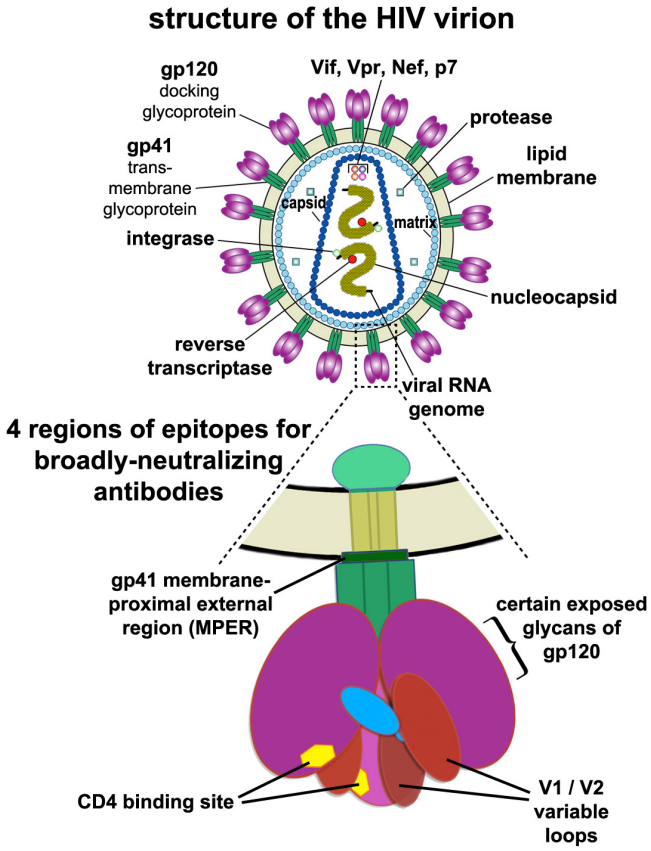


Figure 1.3 Structure of the HIV-1 virion and Envelope trimer

Figure adapted from www.fredhutch.org and B. Haynes et al. (2012) Nature Biotechnology [32]

1.1.6 HIV-1 Envelope Trimer

HIV-1 envelope 'spikes' are comprised of functional gp160 proteins, which are heterodimers that are cleaved into three gp120 molecules non-covalently bound to three gp41 transmembrane molecules. The functional envelope spikes are in trimer formation, which engage with the CD4 receptor on the surface of target cells [33]. Once engaged, the envelope spikes undergo a

series of conformational changes that expose the co-receptor binding site and lead to insertion of the viral capsid into the host cell (Figure 1.4).

However, the non-covalently bound gp120 and gp41 molecules are unstable, leading to the development of a range of functional trimers, non-functional dimers and gp41 'stumps' [34].

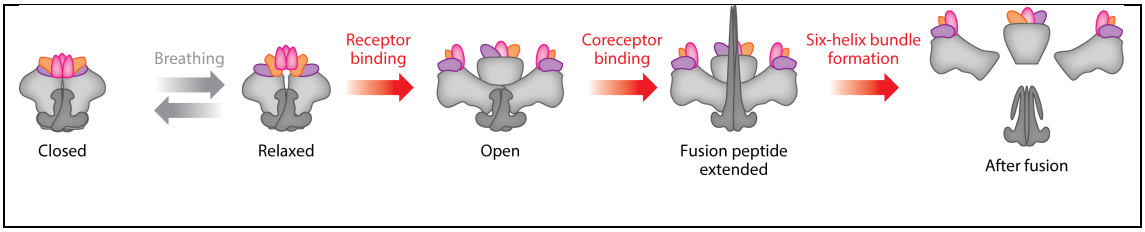


Figure 1.4 Conformations of the Env Trimer.

Before receptor engagement the Env trimers fluctuate between closed, slightly more open, and relaxed conformations. Post CD4 receptor engagement, the trimers open out and the CCR5 (or CXCR4) co-receptor binding sites become available. Co-receptor binding then triggers insertion of the fusion peptide into the host membrane and formation of the six-helix bundle that pulls the host and the virus membrane into fusion proximity. *Adapted from Burton and Hangartner (2016) Annu. Rev. Immunol* [35].

1.1.7 HIV-1 Entry Receptors

CD4, found on the surface of CD4+ T cells, macrophages and dendritic cells (DCs) is the primary receptor for HIV-1. In addition, these cell subsets also express chemokine receptors, CCR5 and CXCR4, co-receptors used by the virus to gain entry to the target cell [36, 37]. CCR5-tropic HIV-1 strains are the primary source of early infection; with CXCR4 viral isolates not arising until after infection has been established [38]. Furthermore, some viral strains are dual-tropic and capable of engaging both co-receptors [39].

Individuals homozygous for the *Δ32-ccr5* allele do not express CCR5 on the cell surface and are subsequently extremely resistant to HIV-1 infection.

Furthermore, *Δ32*-ccr5 heterozygous individuals display a lower CCR5 surface expression and have a slower progression to AIDS [40].

Studies have been performed to determine the levels of CCR5 and CXCR4 expression on CD4+ T cells with results showing that not all T cells have the same levels of CCR5 expression. Naïve and memory CD4+ T cells have low or absent levels of CCR5 expression, which means that they are less susceptible to HIV-1 infection. Conversely, activated T cells have high levels of CCR5 expression, which leaves this CD4+ T cell subset highly vulnerable to HIV-1 infection [41]. However, these studies were performed in PBMC and subsequent analysis of T cells in the genital mucosa of macaques demonstrated that HIV-1 could productively infect naïve T cells in this compartment [42, 43]. Furthermore, studies of SIV (Simian Immunodeficiency Virus) transmission and early infection in the genital mucosa have highlighted that there is a local expansion of infected resting CD4+ T cells that occurs before infection can be detected in the draining lymph nodes and preceding viral dissemination [44].

More recently, it has been demonstrated that some HIV-1 isolates also bind to the gut homing receptor $\alpha_4\beta_7$ integrin [45]. This receptor is found on the surface of gut and vaginal homing T cells [46-48] and binds to the mucosal vascular addressin cell adhesion molecule-1 (MadCAM-1), found in mucosal epithelium. While not a co-receptor used for direct viral infection, $\alpha_4\beta_7$ integrin interacts with the gp120 on the surface of CD4+ T cells potentially providing a means for HIV-1 to attach to target cells, bringing the virus into closer proximity of CD4 and CCR5/CXCR4 [45, 49, 50].

1.1.8 HIV-1 Infection

As mentioned previously, HIV-1 requires the cell surface receptor CD4 [51] and chemokine co-receptors CCR5 or CXCR4 [52] to infect a CD4+ cell. Initially, gp120 interacts with CD4 on the surface of the target cell, which leads to conformational changes in the HIV-1 envelope trimer that exposes the binding site for the co-receptor. Once the co-receptor has bound, fusion peptides are inserted into the cell membrane, ultimately leading to the fusion of the virus to the target cell membrane (Figure 1.5).

1.1.9 HIV-1 Replication Cycle

Post-fusion, the viral capsid is released into the cytoplasm, where it is degraded, revealing the reverse transcription complex, containing the internal viral genome and viral replication enzymes. As HIV-1 is an RNA virus, it must first reverse transcribe its genome into viral DNA to allow integration into the host genome, which it does using reverse transcriptase. The transcribed viral genome and Vpr protein form the pre-integration complex (PIC), which enters the nucleus through the nuclear pore complex without disrupting the nuclear envelope. Once inside the nucleus, the viral DNA is integrated into the host cell DNA using integrase to form a provirus. In activated CD4+ cells, the provirus is transcribed under the influence of transcription factors such as NF-κβ, and translated to produce new viral RNA. However, it is important to note that infection of resting CD4+ cells lacking active transcription factors results in the creation of a latent pool of HIV-1 infected cells that can be reactivated at a later time point [53, 54].

Once transcribed, the new viral RNA and viral proteins are translated and translocated to the cell surface, where the host cell membrane is used to form new immature virions, which bud off and are released. The last stage of the replication cycle is virion maturation, whereby the viral proteases induce the formation of mature Gag proteins. Once this is complete, the virions are fully infectious and ready to infect new target cells [55].

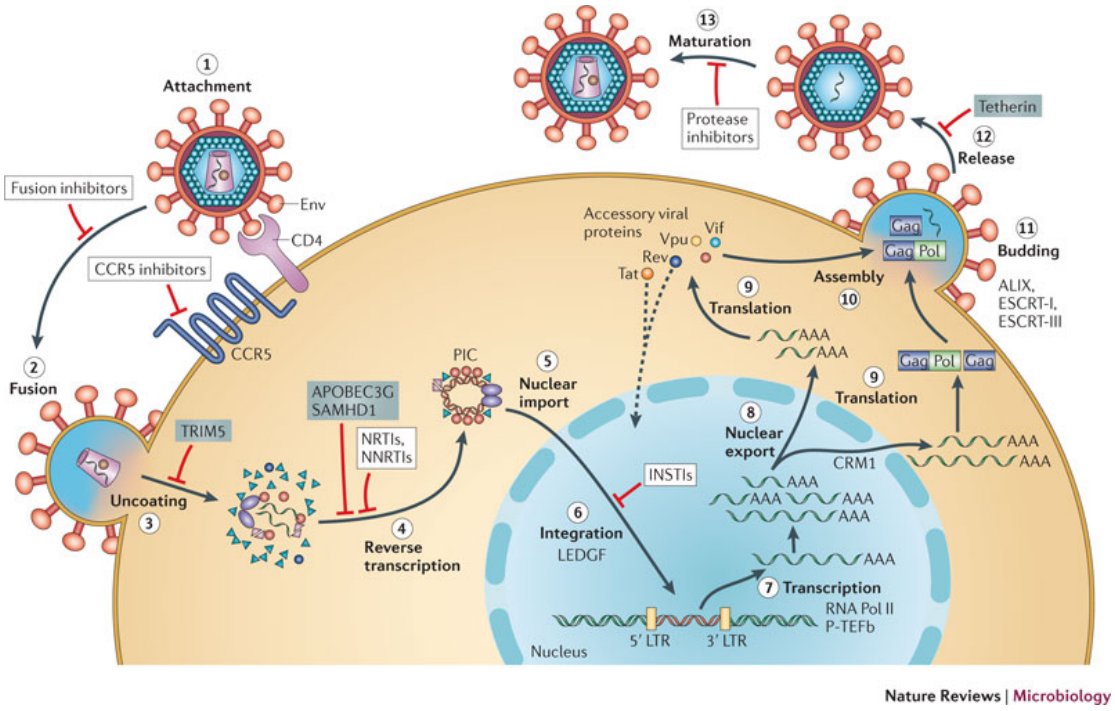


Figure 1.5 Overview of the HIV-1 replication cycle.

Schematic showing the replication cycle of HIV-1, including targets for therapeutic antiviral interventions. INSTI – Integrase strand transfer inhibitor; LTR – long terminal repeat; NNRTI – non-nucleoside reverse transcriptase inhibitor; NRTI – nucleoside reverse transcriptase inhibitor; PIC – Pre-integration complex. *Taken, with permission, from A. Engelman & P. Cherepanov, Nature Reviews Microbiology (2012)* [56].

1.2 HIV-1 Immunology

During the acute phase of HIV-1 infection, the innate immune system attempts to halt the expansion of the virus and inflammatory cytokines are

produced, which induce the early flu-like symptoms in the infected individual. By around day 21, the first adaptive immune responses can be detected, with CD8+ T cells to the original virus in circulation. Shortly after this, the first humoral immune responses can be detected, beginning with non-neutralising antibodies (nnAb) to gp41 at around day 23 and gp120-specific nnAb following at around day 38. However, neutralising antibodies (nAb) do not appear until around 90 days into infection, with broadly neutralising antibodies (bnAb) not arising for many years [57].

1.2.1 Innate Immune System and HIV-1

The innate immune system is the first immunological line of defence against an invading pathogen. The immune response is activated by danger signals found on the surface of pathogenic material such as pathogen-associated molecular patterns (PAMPS) like lipopolysaccharides (LPS), dsRNA and unmethylated CpG motifs. The immune response is fast but non-specific, with no immunological memory initiated. HIV-1 associated PAMPS include structural proteins such as p17, p24 and gp41, which are recognised by TLR2 [58] and ssRNA, recognised by TLR7/8 [59]. However, HIV-1 has evolved mechanisms to evade or subvert the innate immune system, enabling the virus to gain entry to target cells without triggering an immune response [60, 61].

1.2.1.1 Dendritic Cells

Dendritic Cells (DCs) are professional antigen-presenting cells of the innate immune system, constantly sampling their local environment to detect any invading pathogens. Immature DCs are particularly efficient at taking up

antigen via pattern recognition receptors (PRR). Once they have captured antigen, they migrate to lymphoid tissues where they mature and upregulate MHC class II molecules becoming efficient T cell stimulators [62].

DCs express CD4 and HIV-1 co-receptors, CCR5 and CXCR4, on their surface, and are therefore capable of becoming directly infected with HIV-1 [63-65]. There are three main subtypes of DCs, CD1c+ myeloid DCs (mDC) and CD141+ mDC, which also express CD11c, and CD303+ plasmacytoid DCs (pDC), which also express CD123 [66].

DCs can initiate infection of CD4+ T cells via one of two routes: *cis*-infection and *trans*-infection [67]. While HIV-1 replication within DCs is relatively low, *cis*-infection involves the *de novo* replication of DCs and subsequent onward infection of target cells. Cell-to-cell contact is a more efficient mode of HIV-1 transfer from DC-to-CD4+ T cells and this is achieved via *trans*-infection. Here, the DCs do not become productively infected, but infect CD4+ T cells via an infectious synapse mediated by DC-SIGN-CD4 T cell receptor interaction [68]. Furthermore, *trans*-infection can be mediated through viral endocytosis and release of the virus in exosomes [69].

DCs express Fc receptors (FcR), which allow them to respond by mounting an immune response to antibody-opsonised antigens. Myeloid DCs have been shown to express activating FcR CD16A (FcγRIIIa), CD32A (FcγRIIa), FcγRI (CD64), CD89 (FcαR), as well as inhibitory FcR CD32B (FcγRIIb). Fc-mediated effector functions by DCs include antibody-dependent cellular phagocytosis (ADCP), antibody-dependent cellular cytotoxicity (ADCC) or, for viruses, antibody-dependent cellular viral inhibition (ADCVI) [70, 71]. Furthermore, FcRs on DCs can also act to aid in the internalisation and

subsequent antigen presentation of T cells. In addition, complement receptor expression on DCs, leads to modulation of antigen presentation and regulation of the adaptive immune response [72]. Fc- and complement receptors and their associated effector functions are further discussed in later sections.

1.2.1.1.1 Langerhans Cells, Langerin and HIV-1

Langerhans cells (LC), a subset of mDC, reside in the stratified squamous epithelium of the skin, oral and genital mucosa and are ideally placed to survey mucosal surfaces [73-75]. LCs can be distinguished from other DC subsets by the high expression of CD1a, as well as the C-type lectin, Langerin (CD207). Capture of non-opsonised HIV-1 gp120 by Langerin leads to internalisation and degradation of the virus within Birbeck granules inside the cells, preventing onward transmission of the virus [76, 77], although this antiviral function can be overwhelmed by high viral loads. Furthermore, a recent publication has demonstrated that HIV-1 infection is restricted by tripartite-containing motif 5α (TRIM5α) in Langerin-positive LC, but not DC-SIGN+ DCs [78]. LCs have also been implicated in the capture and subsequent transfer of HIV-1 to CD4+ T cells within mucosal tissue [79].

1.2.1.1.2 Dendritic Cells, DC-SIGN and HIV-1

Interstitial DC (iDC), found in the dermis, oral, vaginal and colonic lamina propria express a different C-type lectin on their surface, DC-specific intercellular adhesion molecule-3-grabbing non-integrin, DC-SIGN (CD209). Unlike LCs, which only efficiently capture antigen when in their immature state, iDCs can take up antigen irrespective of their maturation status and are extremely efficient at transferring HIV-1 to T cells through *trans*-infection [80].

Like Langerin on LCs, DC-SIGN complexes with the gp120 on the surface of HIV-1 but, whereas Langerin may have a function in protecting cells from infection, DC-SIGN has been shown to protect the virus from degradation [81]. However, colorectal mucus has been shown to interfere with DC-SIGN, preventing *trans*-infection of CD4+ T cells [82]. Furthermore, it has been shown that antibody-opsonisation of HIV-1 by nnAb significantly reduces the internalisation of virions by DCs and also impairs subsequent *trans*-infection of CD4+ T cells [83].

1.2.1.1.3 Mannose-binding Lectin and HIV-1

Langerin and DC-SIGN are the C-type lectins most commonly associated with HIV-1, but they are not the only lectins capable of interfering with HIV-1 infection. Mannose-binding lectin (MBL) has also been shown to interact with gp120, disrupting the ability of the virus to engage entry receptors in a non-complement dependent manner [84, 85].

1.2.1.2 Macrophages

Macrophages share a common myeloid progenitor cell within the bone marrow that can develop into DCs, neutrophils, eosinophils, basophils and mast cells [86]. They patrol the body acting as innate immune scavengers by phagocytosing cellular debris, cancerous cells, foreign substances, infected cells and invading pathogens. They also have a role in wound healing and antigen-presentation and are found throughout the body, including mucosal surfaces [87]. Once internalised, unwanted material is degraded within phagolysosomes by enzymes and reactive oxygen species (ROS) [88].

Macrophages express CD4 and chemokine receptors CCR5 and CXCR4, making them a target cell for HIV-1. Although usually not infected by T cell-

tropic (T-tropic) transmitter/founder viruses, they are targets during the later stages of infection [89]. Macrophage-tropic (M-tropic) HIV-1 viruses evolve to specifically infect cells with low levels of CD4, such as macrophages, and are often found within the central nervous system (CNS) as both R5 and X4 viruses [90-92].

In the context of HIV-1 infection, macrophages can transmit the virus to bystander CD4+ T cells, become productively infected and be induced to phagocytose infected cells via antibody- and complement-dependent mechanisms [93, 94].

HIV-1 infection of macrophages by cell-free virions is limited due to low expression of the entry receptors, but has been shown to occur [68, 95, 96]. *In vitro* experiments have shown that macrophages are most commonly infected through the phagocytosis of infected T cells or through direct cell-to-cell spread [68, 97]. Indeed, a recent *in vitro* study investigating the cell-to-cell spread of HIV-1 from infected CD4+ T cells to macrophages observed novel virological synapse formation between monocyte-derived macrophages and CD4+ T cells that is distinct from other synapse formations [98]. However, a recent *in vivo* study using humanised mouse models demonstrated that macrophages could be infected in the absence of infected T cells [99].

An important feature of macrophages is the expression of Fc- and complement receptors that, through the process of opsonisation, assist in the recognition and destruction of pathogenic material. Macrophages have been shown to express activating FcR CD16A (FcγRIIIa), CD32A (FcγRIIa), CD64 (FcγRI), CD89 (FcαR), as well as inhibitory FcR CD32B (FcγRIIb), capable of interacting with antibody-opsonised pathogens [100, 101]. Interaction with

opsonised pathogens leads to destruction through ADCP, ADCC or, for viruses, ADCVI [101]. Furthermore, complement-opsonisation of pathogens and interaction with complement receptors on macrophages leads to removal of the infecting pathogen through a process known as complement-dependent cytotoxicity (CDC) [102]. FcRs, complement, ADCP, ADCC, ADCVI and CDC are all discussed in depth later on in this introduction.

1.2.1.3 Natural Killer Cells

Natural killer (NK) cells are lymphoid-derived cells and the principle innate effector cells to respond to virally-infected or tumour cells [103, 104]. They have been shown to be present within mucosal tissues associated with HIV-1 infection and play a role in preventing HIV-1 transmission [105-107]. Once virally-infected cells have been encountered, cytolysis is achieved through activating NK cell receptors, NKG2D, leading to either release of cytotoxic granules containing granzymes and perforin, which destroy the infected cells or induction of apoptosis of infected cells through intracellular signalling [108]. Moreover, NK cells can bridge the gap between the innate and adaptive immune systems by engaging antibody, through FcR (CD16A (FcγRIIIa)), leading to ADCC [109, 110]. Furthermore, despite being part of the innate immune system, recent investigations in mice and NHP have highlighted the potential for NK cells to develop antigen-specific immunological memory [111, 112].

NK cell activity is regulated by inhibitory receptors, which recognise major histocompatibility complex (MHC) class I molecules and prevent NK cells from exerting their cytotoxic activities on uninfected or untransformed cells. These inhibitory receptors are either in the form of killer immunoglobulin-like receptors (KIR) or CD94/NKG2A, both of which recognise different variants of

human leucocyte antigen (HLA) molecules. HIV-1 has evolved methods to subvert NK cell immune responses to virally infected cells including selective up- or downregulation of HLA alleles, inhibition of activating NK cell receptors and modulation of cytokine expression [110, 113, 114].

NK cells express HIV-1 co-receptors CCR5 and CXCR4, but the majority are CD4-negative. However, a small population of NK cells have been observed to express CD4, as well as the co-receptors CCR5 and CXCR4. Investigations into the infectivity of this NK cell subset have demonstrated these cells can become productively infected with HIV-1 [115-117].

1.2.1.4 Neutrophils

Neutrophils are polymorphonuclear leucocytes that represent between 50-75% of the circulating leucocyte population, making them the most abundant white blood cell. Through the use of PRRs, their primary function is to rapidly recognise and phagocytose foreign microorganisms, killing them via production of reactive oxygen species (ROS), proteolytic enzymes or neutrophil extracellular traps (NETs) [118].

Neutrophils express CD89 (FcαR), CD16B (FcγRIIIb), CD32A (FcγRIIa), CD32B (FcγRIIb) and CD64 (FcγRI) on the surface and have been observed to perform antibody-dependent effector functions such as ADCC and ADCP [119].

During the course of HIV-1 infection, neutrophil-loss or neutropenia is observed in 35-75% of infected individuals. Interestingly, it has been demonstrated that HIV-1 can enter into the bone marrow and infect precursor cells that reside there. Loss of neutrophils in HIV-1 infected individuals can lead to increased susceptibility to bacterial and fungal infections [120].

1.2.1.5 The Complement System

The complement system is comprised of cell-bound and plasma-based proteins that function to modulate and enhance the innate or adaptive immune responses through three different pathways. The classical pathway is activated through complement components binding with high avidity to the Fc-portion of an antibody-opsonised antigen or infected cell. The alternative pathway is triggered when complement protein C3b binds to a pathogen or infected cell. Lastly, the mannose-binding lectin (MBL) pathway is triggered when complement components interact with mannose-binding lectin-opsonised pathogens or infected cells [121]. Regardless of the pathway, a complement cascade is initiated that usually concludes with the formation of the membrane attack complex (MAC), causing lytic holes in the infected cell membrane and culminating in the death of the cell in a process known as complement-dependent cytotoxicity (CDC) [121]. In addition, complement can act through complement receptors to induce complement-mediated phagocytosis and is also capable of attracting macrophages and neutrophils to the site of infection [122].

The role of complement in HIV-1 infection is complex, with some studies indicating that it may have a protective role in early HIV-1 infection [123-125] and also in elite controllers [126]. However, HIV-1 has evolved a number of mechanisms to evade complement activity and may even use the complement system to enhance infectivity of target cells [127-129].

1.2.2 Fc Receptors (FcR)

Fc receptors (FcR) recognise and bind to the Fc-portion of antibodies and, in doing so, bridge the gap between the innate and adaptive immune systems by

enabling innate immune cells to recognise and act against antibody-opsonised pathogens. FcR are found on the surface of all innate immune cells in either inhibitory or activating forms (Figure 1.6).

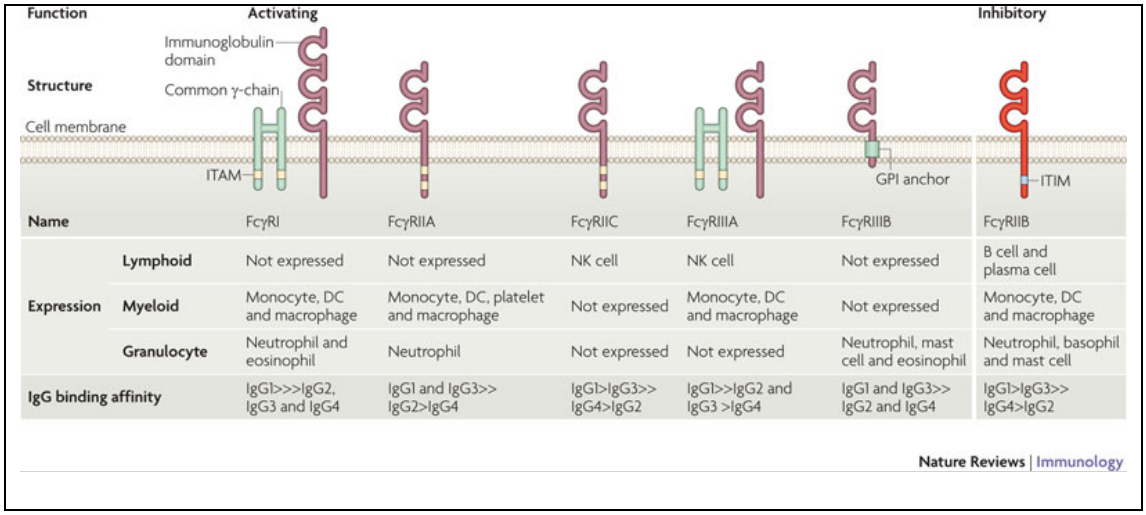
Inhibitory FcR (CD32B) are found on the surface of B cells, as well as macrophages, DCs and granulocytes. Their activity is governed by the presence of an immunoreceptor tyrosine-based inhibitory motif (ITIM) transmembrane domain. Once internalised the immune-antigen complex is preserved intact for transfer to B cells. The main function of the inhibitory receptor is a regulatory one through modulation of the B cell responses to limit the development of autoreactive B cells [130, 131].

The majority of FcR are activating, signally through the immunoreceptor tyrosine-based activating motif (ITAM) transmembrane domain. Analogous to the inhibitory FcR, they internalise immune-antigen complexes, but the final outcome of the internalisation is degradation leading to antigen processing and presentation or the initiation of further downstream effector functions, such as ADCC, ADCP or ADCVI [131, 132].

FcγR differ in their ability to bind individual IgG subclasses, with IgG1 more likely to interact with CD64 (FcγRI) and IgG3 more likely to bind to CD16A (FcγRIIIa) [133]. Indeed the induction of IgG3 antibodies and the subsequent effector functions are postulated to be one of the key correlates of protection in the RV144 vaccine trial (discussed further in section 1.4.3.2) [134].

Genes encoding for FcR can lead to polymorphic variants of activating receptors CD16A (FcγRIIIa) and CD32A (FcγRIIa), which affects their ability to bind to IgG subclasses. Allelic variants of CD16A (FcγRIIIa) include two variants with polymorphisms at position 158, leading to variants FcγRIIIa$_{V158}$ and

FcγRIIIa$_{F158}$. Similarly, allelic variants within CD32A (FcγRIIa) are caused by polymorphisms at position 131, leading to two variants: FcγRIIa$_{R131}$ and FcγRIIa$_{H131}$. All allelic variants bind equally to IgG1 and IgG3, but FcγRIIa$_{H131}$ binds with higher affinity to IgG2 when compared to FcγRIIa$_{R131}$ and FcγRIIIa$_{V158}$. IgG4 binds with high affinity to all FcR, with the exception of FcγRIIIa$_{F158}$, which binds at a lower affinity [133]. Allelic variants have been proposed as another correlate of protection in the RV144 trial [135, 136] and are additionally thought to be of relevance in the immune response to certain cancers and autoimmune diseases [137, 138].



Name		FcγRI	FcγRIIA	FcγRIIC	FcγRIIIA	FcγRIIIB	FcγRIIB
Expression	Lymphoid	Not expressed	Not expressed	NK cell	NK cell	Not expressed	B cell and plasma cell
	Myeloid	Monocyte, DC and macrophage	Monocyte, DC, platelet and macrophage	Not expressed	Monocyte, DC and macrophage	Not expressed	Monocyte, DC and macrophage
	Granulocyte	Neutrophil and eosinophil	Neutrophil	Not expressed	Not expressed	Neutrophil, mast cell and eosinophil	Neutrophil, basophil and mast cell
IgG binding affinity		IgG1>>>IgG2, IgG3 and IgG4	IgG1 and IgG3>> IgG2>IgG4	IgG1>IgG3>> IgG4>IgG2	IgG1>>IgG2 and IgG3 >IgG4	IgG1 and IgG3>> IgG2 and IgG4	IgG1>IgG3>> IgG4>IgG2

Figure 1.6 Fc Receptors – cellular expression and IgG binding affinity.
Taken from K. Smith and M. Clatworthy – Nature Reviews Immunology (2010) [139].

1.2.2.1 CD16 (FcγRIII)

CD16A (FcγRIIIa) is the low/intermediate affinity IgG receptor, predominantly expressed by NK cells, but also found on monocytes, macrophages and DCs and most commonly associated with ADCC [132, 140]. Meanwhile, CD16B (FcγRIIIb) is expressed by neutrophils and is most commonly

associated with phagocytosis. CD16B is different in structure to other FcR in that it is linked to the plasma membrane through a glycosylphosphatidylinositol (GPI) anchor [141].

CD16 expressing cells have been found within mucosal tissues, indicating that they may be able to mount a response to vaccine-induced antibody [142-145].

CD16 preferentially binds to the IgG3 subclass. Indeed, induction of this antibody subclass in the RV144 vaccine trial and subsequent effector functions are thought to be some of the correlates of protection [134].

1.2.2.2 CD32 (FcγRII)

CD32 (FcγRII) is the low affinity receptor for IgG and can function as an activating (CD32A and CD32C) or inhibitory (CD32B) receptor, depending on the transmembrane ITAM or ITIM domain. Activating CD32A is predominately expressed on monocytes, macrophages, DCs and neutrophils, whereas CD32C is predominantly found on NK cells. Inhibitory CD32B is expressed on the same cell subsets as CD32A, with the addition of expression on B cells and plasmablasts [146]. Overall, CD32 is most broadly expressed FcγR on immune effector cells found in the blood [147].

As mentioned, the inhibitory receptor CD32B modulates the B cell response via a feedback loop. Its primary function is to limit the production of autoreactive B cells [148]. Effector functions of the activating receptor, CD32A, are initiated by the cross-linking of FcR-antibody complexes, leading to cell lysis and/or phagocytosis [149, 150].

CD32 expressing cells are found throughout the body, including mucosal surfaces associated with HIV-1 transmission, indicating a potential role in

vaccine-induced antibody responses. Interestingly, it has been recently observed that antibodies from HIV-1 controllers and untreated progressors exhibit increased phagocytic activity, altered Fc domain glycosylation, and skewed interactions with CD32A and CD32B in both bulk plasma and HIV-specific IgG [151].

1.2.2.3 CD64 (FcγRI)

CD64 (FcγRI) is the high affinity FcR for IgG and is the only FcR that can bind to monomeric IgG in addition to immune complexes. It is constitutively found on the surface of macrophages and DCs, while its expression can be induced on mast cells, eosinophils and neutrophils by cytokines such as IFN-γ and IL-10. Interestingly, IFN-γ expression also increases the affinity of monocyte-derived CD64 for immune complexes [146, 152-154]. CD64 expression within tissue has been observed, largely due to its expression on macrophages, although investigations into expression within mucosal tissues are more limited [155].

CD64 associates most avidly with IgG1 and IgG3 followed by IgG4, demonstrating no ability to bind to IgG2 [146]. Engagement of the receptor by monomeric IgG or immune complexes culminates in phagocytosis of the target cell, release of inflammatory cytokines, production of ROS and/or cross-presentation of the antigen to T cells [156].

1.2.2.4 CD89 (FcαR)

CD89 (FcαR) is the FcR for IgA and is expressed by macrophages, DCs, neutrophils, as well as platelets and NK cells [157]. CD89 expression on NK cells can be upregulated in the presence of TNF-α [158, 159]. Expression of CD89

within tissue is largely depended on the presence of neutrophils and macrophages but it has been observe in gastrointestinal tract and skin [160].

Activity is induced through CD89 binding of aggregated IgA complexed with antigen leading to phagocytosis and ADCC [100].

1.2.2.5 Neonatal Fc Receptor (FcRn)

Initial findings determined that the FcRn functioned to transport IgG across the placenta from mother to foetus. However, it was subsequently found to have further functions of transcytosis and recycling of IgG [161]. This additional function has increased interest in FcRn as enhanced binding to the receptor has been shown to prolong the half-life of an antibody, subsequently leading to improved antibody function [162]. However, in the context of this thesis, FcRn expression was not investigated.

1.2.3 Antibody-dependent Effector Mechanisms

Once immune complexes bind to activating FcR, a series of effector functions are induced leading to the removal of antibody-complexed antigen through one of a number of mechanisms. These mechanisms are dependent upon a number of parameters, including the FcR engaged and effector cell type induced, as well as immunoglobulin allotype and glycosylation pattern.

1.2.3.1 Antibody-dependent Cellular Cytotoxicity (ADCC)

Antibody-dependent cellular cytotoxicity (ADCC) is initiated most efficiently through the binding of the Fc-portion of an antibody-opsonised cell to CD16A. Furthermore, it can be triggered through binding of CD64 and CD32A on myeloid cells (monocytes, macrophages and DCs) [140, 163].

Monomeric IgG bound to FcγR is not sufficient to initiate ADCC, but instead results in the recycling of the FcγR-IgG complex to the cell surface [164, 165]. For ADCC to occur, the FcγR must bind to a cross-linked IgG antibody complex, which initiates FcγR clustering. Once these events have occurred, ADCC is initiated, which culminates in the release of the contents of cytotoxic granules containing perforin, granzymes and cytokines such as IFN-γ. These cytotoxic agents are released in a directional manner across a lytic synapse leading to cytolysis of the opsonised cells [132].

ADCC-inducing antibodies have been shown to lower the viral set-point in non-human primate (NHP) models of HIV-1 infection [166-169]. Furthermore, studies in humans have indicated that the presence of ADCC antibodies correlate with slower disease progression [167, 170], give rise to significantly lower genital HIV-1 RNA levels in seropositive women [171] and, when found in breast milk, reduce the risk of mother-to-child transmission of HIV-1 [172].

1.2.3.2 Antibody-dependent Cellular Phagocytosis (ADCP)

Antibody-dependent cellular phagocytosis (ADCP) is induced by the binding of the Fc-portion of antibody to an FcR, primarily CD32A, CD64 and CD89, found on the surface of professional phagocytic cells such as monocytes, macrophages, neutrophils and DCs. Upon binding of the antibody-opsonised infected cells or pathogens, FcR cluster and subsequently cross-link, initiating phagocytosis. Once inside the phagocytic cell, the ingested material is degraded by lytic enzymes and ROS [173] [151]. As mentioned previously, ADCC can also be induced through engagement of CD32A and CD64 on myeloid cells [174]. However, the efficiency of this type of ADCC is low when compared to ADCP,

which is reported to occur rapidly and efficiently within 1-4 h, compared to incubation times of up to 24 h for CD32A- and CD64-induced ADCC [175].

1.2.3.3 Antibody-dependent Cellular Viral Inhibition (ADCVI)

Antibody-dependent cellular viral inhibition (ADCVI) is specifically associated with FcγR-induced killing of virally infected cells by FcR effector cells. It is a combination of ADCC and ADCP activity with the additional release of HIV-1 inhibitory β-chemokines from the effector cells, which block onward infection of susceptible cells [176-178].

High ADCVI activity in serum from vaccinated individuals has been demonstrated to inversely correlate with lower rates of sexually acquired HIV-1 infection [179]. Furthermore, ADCVI activity has been shown to inversely correlate with HIV-1 plasma viral load during the acute phase of infection in humans [180], as well as playing a protective role against SIV infection in NHP [181].

1.2.4 Adaptive Immune System and HIV-1

Adaptive immunity is composed of cellular and antibody-mediated immune responses that develop to provide long-lasting, highly specific immunity to a particular pathogen and is the basis for the immunological memory induced by vaccination.

1.2.4.1 T cells

T cells are lymphoid cells of the adaptive immune system that provide cellular immunity in the form of effector and cytotoxic killing functions as well as regulatory and helper functions to aid immune responses from other branches of

the immune system. They originate from haematopoietic stem cells in the bone marrow, but mature within the thymus [182].

T cells can be subdivided into two main groups: CD4+ T cells and CD8+ T cells, with both cell subsets recognising antigen presented by antigen presenting cells via major histocompatibility complex (MHC) molecules to T cell receptors (TCR). In broad terms, CD4+ T cells provide 'help' to other immune cells, whereas CD8+ T cells have cytotoxic functions, particularly in response to viruses.

1.2.4.1.1 CD4+ T cells

CD4+ T cells are activated through presentation of antigenic peptides complexed with MHC class II molecules together with co-stimulatory signals from CD28 on T cells interacting with CD80 or CD86 proteins on antigen presenting cells [183]. Depending on the cytokine environment, the resulting effector CD4+ T cells can be classified into two main categories: T-helper (Th) 1 or Th2 cells. Th1 cells are induced by cytokines such as IL-2 and IL-12, in turn producing inflammatory cytokines, IFN-γ and TNF–α in response to activation. Th2 cells are induced by cytokines such as IL-4, producing regulatory cytokines such as IL-4 and IL-10 in response to activation [184].

A specific subset of T-helper cells that are found in the germinal centres of secondary lymphoid tissue are T-follicular helper (Tfh) cells. These CD4+ T cells are induced by IL-21 and interact with B cells through the expression of CD40L, enhancing their proliferation and survival. Tfh cells are critical for the generation of high affinity antibodies and proliferation of B cells within the germinal centres and are thought necessary for the formation of antibodies in response to vaccination [185].

In the context of HIV-1, CD4+ T cells are the primary target for infection that, over the course of disease, results in depletion of these cell subsets throughout the circulation, although this depletion is particularly severe within the gastrointestinal tract [186]. Moreover, helper Th17 cells and regulatory T cell (Treg) subsets are critical in the maintenance of mucosal barriers. The ratio of proinflammatory Th17 cells to Treg cells is delicately maintained under normal circumstances. However, during HIV-1 infection Th17 cells are preferentially infected and are particularly prone to depletion within mucosal surfaces [187-189]. Furthermore, regulatory T cells, important for maintaining peripheral tolerance and regulating immune activation, are found in elevated numbers in circulating blood of chronically infected HIV-1 patients, albeit with decreased total Treg cells throughout the individuals. It has been postulated that the elevated Treg numbers found within the circulating blood of chronic HIV-1 patients could have an impact on HIV-1 specific immune responses and reduce immune activation [190-193].

1.2.4.1.2 CD8+ T cells

CD8+ T cells are activated in a similar way to CD4+ T cells, however, antigenic peptides are instead complexed with MHC class I molecules. Upon activation, CD8+ T cells secrete inflammatory cytokines IFN-γ, TNF-α and IL-2, as well as perforin and granzymes [194].

CD8+ T cells are critical in the immune response to viruses, with strong responses to HIV-1 detected in the acute phase of infection. However, the virus is not controlled by CD8+ T cell responses due to the development of escape mutants and downregulation of MHC class I on the surface of infected cells [195,

196]. During the course of HIV-1 infection, continued high level stimulation and proliferation of CD8+ T cells eventually leads to exhaustion of the cell subset, impacting on its ability to mount an effective immune response [197]. Moreover, memory CD8+ T cell responses are thought to be an important outcome of an effective vaccine.

Human leucocyte antigens (HLA) are the human MHC molecules and they play a key role in the immune response to antigens. Allelic variations within individuals leads to differences in the ability to present certain antigens to CD8+ T cells and consequently impacts on the strength of the immune response. In HIV-1 infection, HLA alleles B27 and B57 are associated with slower disease progression, whereas B35 and B45 are associated with a faster disease progression [198].

1.2.4.2 B cells

B cells are lymphoid cells of the adaptive immune system that produce antibody and also act as antigen-presenting cells. They develop and mature in the bone marrow, where VDJ recombination occurs during the very early stages of B cell development. It involves rearrangement of the V (variable), D (diversity) and J (joining) sections of the immunoglobulin heavy and light chains (V and J, only) and produces a wide repertoire of B cells of different specificities that are released from the bone marrow and migrate to the secondary lymphoid tissue [199].

1.2.4.2.1 B cell Activation

Once inside the secondary lymphoid tissue, activation occurs when the B cell receptor (BCR) interacts with its corresponding antigen in either a T cell-independent or –dependent manner [199].

T cell-independent activation happens when BCR encounter antigens associated with bacterial or viral pathogens, such as LPS (a particularly good activator) or unmethylated CpG DNA, and leads to the production of relatively low affinity antibody [200].

Conversely, T cell-dependent activation produces antibody of higher affinity. Activation occurs when BCR engage and endocytose antigen, ultimately leading to presentation of the constituent peptides via MHC class II molecules on the surface of B cells to TCR on CD4+ T-helper or CD4+ T-follicular helper cells. The CD4+ T-helper cells express CD40L and produce cytokines such as IL-21, which are essential co-stimulatory responses required by B cells to initiate proliferation, antibody production, class-switching and somatic hypermutation [201, 202]

1.2.4.2.2 Antibodies

Human antibodies are found in five different isotype forms: IgM, IgD, IgG, IgA and IgE. Each isotype is comprised of two heavy (H) and two light (L) immunoglobulin chains, connected through disulphide bonds (Figure 1.7). The CH2 and CH3 domains form the Fragment, Crystallisable (Fc)-region, which contains binding regions for Fc- and complement receptors, leading to the downstream effector functions previously described. The VH and VL domains form the Fragment, Variable (Fv)-region, which is the section of the antibody that binds antigen. The antigen binding section of the VH and VL domains are

comprised of three complementarity-determining regions (CDR). Finally, the CH1, CL, VH and VL sections combine to form the Fragment, Antigen binding (Fab)-region, which binds antigen, but also gives the antibody a level of flexibility [203].

Under normal circumstances IgG is the most commonly occurring isotype in the blood, followed by IgA, IgM, IgD and IgE [204]. All isotypes are found in monomeric form, however IgA and IgM are also found in dimeric and pentameric forms, respectively. IgA and IgM form their respective structures through association with the J chain [205]. Furthermore, IgA is often found in association with the secretory component, which enables the immunoglobulin to transcytose into mucosal compartments, making IgA the most abundant isotype within mucosal secretions [206].

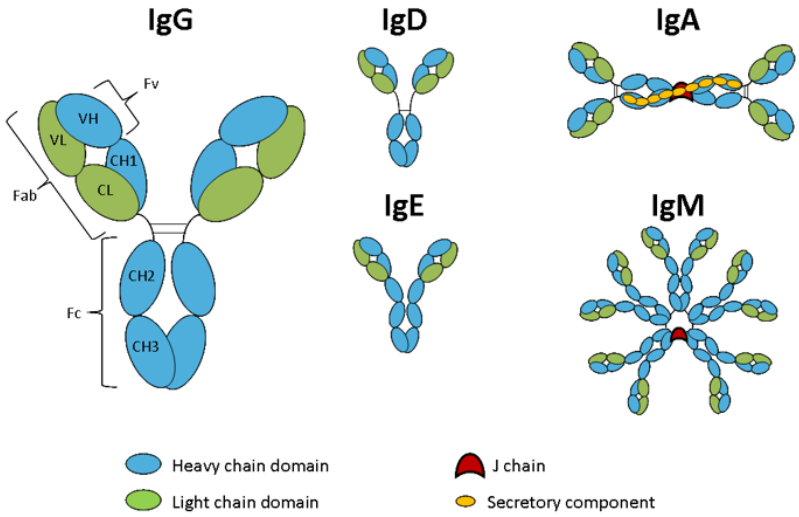


Figure 1.7 Immunoglobulin Isotypes

Image taken from www.absoluteantibody.com

Moreover, immunoglobulin isotypes IgG and IgA can be subdivided into subclasses: IgG1, IgG2, IgG3, and IgG4, IgA1 and IgA2. The subclasses differ in their constant and hinge regions and number of disulphide bridges between the two heavy chains, which alter their ability to interact with FcR and antigen

(Figure 1.8) [133, 207]. Indeed, one of the proposed correlates of protection in the RV144 vaccine trial was IgG3 production and associated ADCC effector response [134]. T cell independent activation by polysaccharides tend to induce class-switching to IgG2, whereas T cell dependent activation is more likely to induce IgG1 or IgG3 [208].

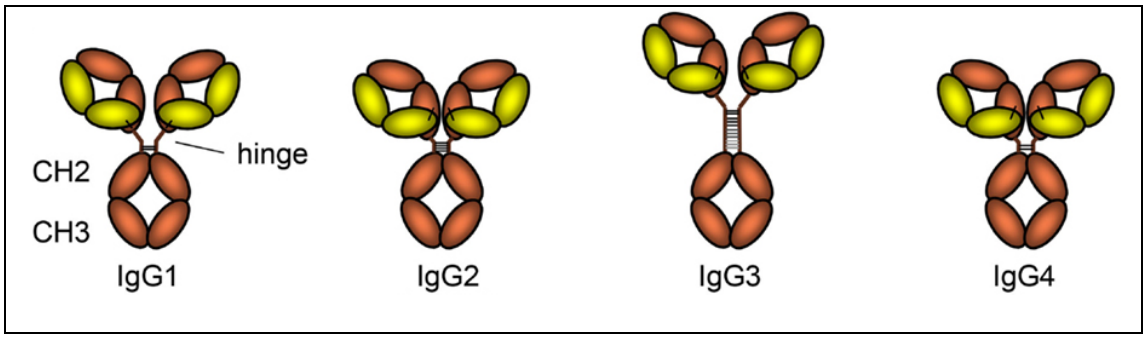


Figure 1.8 IgG Subclasses

Modified from G. Vidarsson et al (2014) Front. Immunol [208].

1.2.4.2.3 Class-Switching

The first antibodies to be produced by naïve B cells are isotypes IgM and IgD, but once the BCR recognises antigen and B cells begin to proliferate, these isotypes switch to IgG, IgA or IgE through a series of gene rearrangements within the constant regions of the immunoglobulin [201].

1.2.4.2.4 Affinity Maturation and Somatic Hypermutation

Affinity maturation occurs in the germinal centres within secondary lymphoid tissue and is the process by which antibodies enhance their affinity and avidity for their target antigen. Antibodies achieve this through a combination of somatic hypermutation (SHM) and clonal selection with the aid of T-follicular helper (Tfh) cells [202, 209, 210].

SHM occurs in the dark zone of the germinal centre and is associated with high rate of B cell expansion and subsequent point mutations, leading to

rearrangement of the variable genes within immunoglobulins [211]. As B cells quickly proliferate in response to antigen, there is an increase in the number of mutations within the variable region, some of which may lead to increased affinity for the target antigen. These mutated B cells leave the dark zone and transit into the light zone of the germinal centre, where they interact with antigen presented by Tfh cells or follicular DCs. The higher the affinity of the BCR for the presented antigen, the more antigens are internalised and the greater the co-stimulatory feedback from the Tfh cells. This leads to positive selection of higher affinity BCR, which cycle back to the dark zone [212]. This process of SHM and clonal selection repeats and produces antibodies with affinities that are significantly higher than the original BCR.

1.2.5 Antibody Production During HIV-1 Infection

The main objective of HIV-1 vaccine design is to produce a vaccine capable of eliciting broadly neutralising antibodies (bnAb) that offer cross-clade protection. During the natural course of HIV-1 infection the incidence of bnAb is low, with ~10% developing high levels of bnAb capable of inhibiting 90% of viruses and ~50% of HIV-1 infected individuals develop neutralising antibodies (nAbs) capable of inhibiting more than 50% of viral isolates [213, 214]. Importantly, even in individuals who develop bnAb, viral escape means that there remains continued infection [215]. Conversely, all HIV-1 infected individuals develop nnAb from the early acute phase of infection onwards [216, 217]. Antibody development from nnAb to bnAb is not a linear process, and it is important to note that these antibodies are found as a spectrum within infected individuals [218].

1.2.5.1 Neutralising Antibodies (nAb)

Neutralising antibodies (nAb) inhibit HIV-1 infection by binding to the trimeric envelope spike, blocking key epitopes on the surface of the virus and preventing it from engaging with its entry receptors [219]. However, prior to binding to the host cell these epitopes are largely occluded by the heavily glycosylated glycan shield, leading to limited opportunity for antibodies to develop [220]. BnAb have been discovered against the CD4-binding site, gp41 Membrane Proximal External Region (MPER) and various glycan-dependent epitopes on gp120 (Figure 1.9). BnAb to HIV-1 are unusual in that they have high levels of SHM, combined with long heavy chain complementarity determining region 3 (HCDR3) [35, 221], character traits often associated with self-reactive B cells that are prone to clonal deletion [220, 222]. Indeed, certain HIV-1 bnAb have been shown to have autoreactive and/or polyreactive properties [223]. Autoreactivity and clonal deletion pose problems for induction of bnAb via vaccination. Furthermore, immunodominant nnAb epitopes mean that antibodies are more likely to develop to these over less dominant bnAb epitopes [34]. Lastly, early germline B cell receptors (BCR) have been shown to poorly recognise HIV-1 Env [224]. Recent studies using humanised mouse models have demonstrated that sequential immunisations in transgenic mice with specially designed immunogens could be used to drive these early germline BCR toward development of bnAb [225-228]. While these studies are in the early stages, they represent the most encouraging results to date with regards to the development of a vaccine to induced bnAb in humans.

Several passive immunisation studies using nAb in macaques have shown topical application of nAb to be effective at preventing simian-human

immunodeficiency virus (SHIV) infection [166, 168, 229]. More recently, human phase I clinical trials designed for therapeutic use and involving passive infusion of CD4bs-specific bnAb into infected participants have demonstrated the antibodies to be well tolerated as an infusion [230], with their administration leading to reduced viraemia [231, 232]. These studies highlight the potential for use of passive infusion as a therapy or in HIV-1 prevention.

Furthermore, two antibody-mediated protection (AMP) phase II clinical trials have recently begun in North and South America and Africa investigating intravenous infusion of the CD4bs-specific bnAb, VRC01 [233, 234]. The placebo-controlled AMP trials investigate high-risk HIV-1 negative participants infused with either 10 mg/kg or 30 mg/kg intravenous VRC01 every 8 weeks with the goal to determine the prevention of HIV-1 acquisition.

In addition to classical neutralisation, it has been demonstrated that nAb also exert their activity through engagement of FcR. A NHP study observed that, by mutating the Fc region of nAb, a reduction in ability to prevent SHIV infection followed. Significantly, the inhibitory ability of the nAb was not completely abolished [235]. This suggests that nAb have the potential to function through both a combination of both Fab and Fc-interactions, indicating that modification of the Fc region of nAb could have potential for increasing their function. Furthermore, interactions with FcRn have been shown to increase the half-life of SHIV-specific antibodies *in vivo* [236].

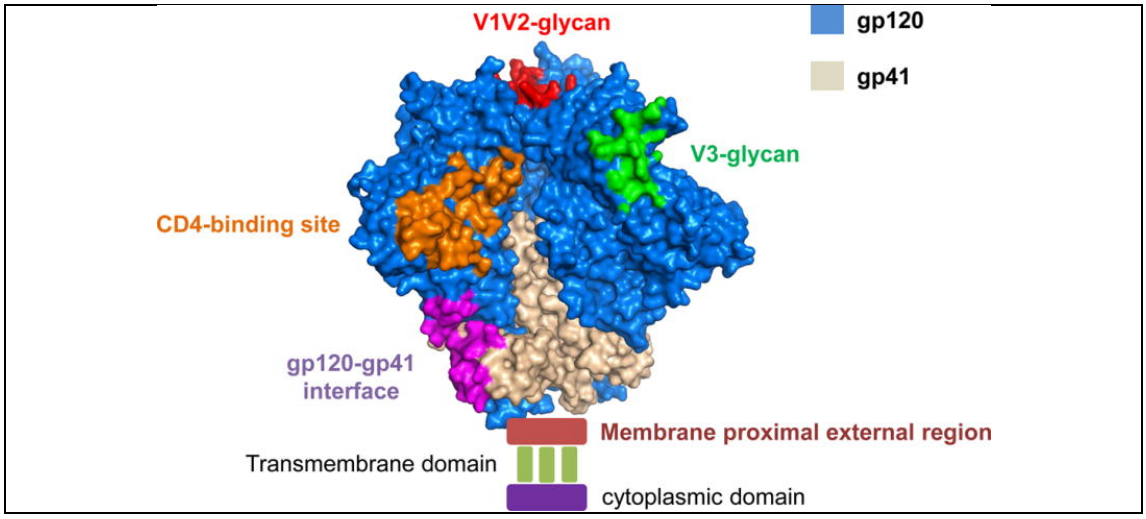


Figure 1.9 HIV-1 trimer and associated broadly neutralising antibody binding sites.

Taken from B. Haynes et al. Cell Host Microbe (2016) [237]

1.2.5.2 Non-neutralising Antibodies (nnAb)

In contrast to bnAb, which may take years to develop, nnAb are found in circulation from the acute stages of infection onwards [216]. NnAb activity is delivered through Fc-mediated effector functions, such as ADCC, ADCP and ADCVI. As previously described, HIV-1 envelope glycoproteins shield neutralising epitopes, which are only revealed once binding of the virus to the CD4-receptor induces conformational changes in the envelope structure [238]. Furthermore, HIV-1 envelope has been observed as a mixture of functional trimers, dimers, monomers and gp41 stumps that have shed all of their gp120 [34]. This diversity of available epitopes leads to antibody development against all forms of the envelope, albeit of a non-neutralising nature.

Interest in inducing nnAb responses through vaccine design has increased since the results from the RV144 vaccine trial (described in full in section 1.4.3.3). Although, nnAb have so far not been able to prevent infection in NHP models, they have demonstrated an ability to lower viral set-point post-infection and limit the number of transmitter/founder viruses [166, 239, 240], indicating a

role for Fc-mediated effector functions in HIV-1 transmission and infection. Furthermore, during the natural course of infection, nnAb and associated Fc-mediated effector functions have been shown to play a role in viraemic control, as well as slowing disease progression and reducing rate of mother-to-child transmission [126, 151, 170, 172]. Conversely, impairment of nnAb effector functions has been demonstrated to advance disease progression [71].

Antibody subclass and glycosylation can also have an impact on the function and protective capacity of nnAb. IgG1 and IgG3 antibodies have been associated with control during natural infection and in vaccine-induced responses [126, 134]. Glycosylation of the Fc-region of an antibody can modulate the ability of nnAb to interact with FcR, with studies demonstrating enhanced FcR binding and increased effector functions in Fc-glycan modified antibodies [151, 241, 242].

However, a recent study highlighted the ability of ADCC-inducing nnAb to enhance uninfected bystander CD4+ T cell depletion by selectively targeting these cells over infected CD4+ T cells, an observation that should be taken into consideration for vaccine-induced nnAb to HIV-1 [243].

1.3 Mucosal Surfaces and HIV-1 Acquisition

The majority of HIV-1 transmissions via mucosal surfaces occur during sexual activity [244, 245]. Regardless of the mucosal surface, HIV-1 must gain entry through the mucosal epithelium, go on to create a small foci of productively infected cells within the subepithelium and finally be transported to the lymph nodes, where systemic infection is initiated [246]. As mucosal surfaces are the primary sites of HIV-1 infection, it is important to have an

understanding of the cells that are present within these tissues, both in terms of early transmission events and from an immunological point of view (Figure 1.10).

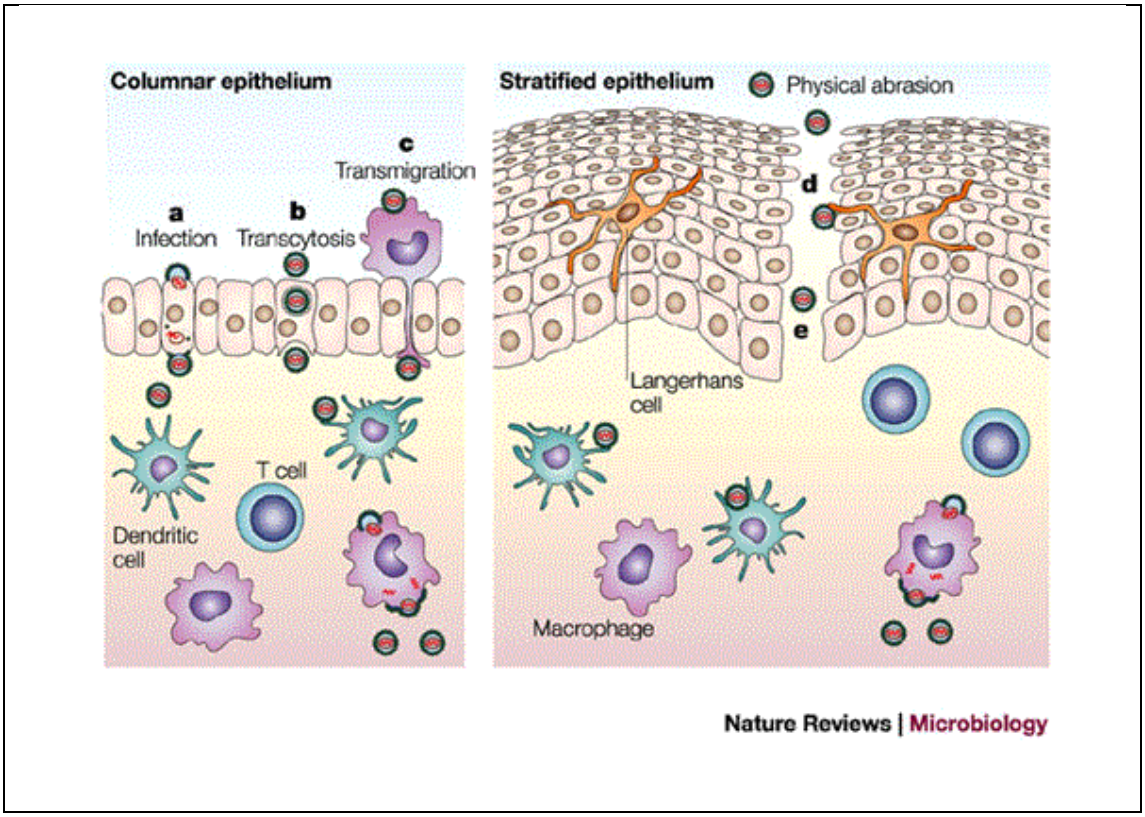


Figure 1.10 Potential Mechanisms of HIV-1 Transmission across Mucosal Surfaces.

a) Direct infection of mucosal epithelial cells; b) Transcytosis through epithelial cells; c) Transmigration through epithelial cells; d) Uptake by Langerhans cells or DCs through engagement with C-type lectins; e) Entry through disrupted epithelium.

Diagram taken from R. Shattock & J. Moore Nature Reviews Microbiology (2003) [247].

In addition to mucosal surfaces, HIV-1 can also be transmitted through transfusion of blood or blood products, intravenous drug use, occupational exposure and mother-to-child transmission (Table 1.1). Risk factors that increase the chances of HIV-1 infection occurring include high viral load, additional pre-existing sexually transmitted infections (STIs) and inflammatory conditions at transmission sites e.g. Bacterial Vaginosis [248-250]. While HIV-1 has been shown to be efficiently transmitted via cell-to-cell spread *in vitro* [94, 251-253], the contribution of this mode of transmission *in vivo* remains to be

fully determined. Indeed, during the early days of the HIV-1 pandemic, virally infected blood products were given haemophiliacs, which resulted in the seroconversion of 60-80% of these individuals [254, 255]. Critically, the plasma-derived factor VIII they were given was cell-free, indicating this mode of viral transmission may also be a major contributing factor during the early stages of HIV-1 transmission.

Table 1.1 Estimated per-act HIV Transmission Risk

	Exposure Route	Risk per 10000 exposures to infected source
Parental Exposure	*Blood Transfusion*	9250
	Needle-sharing injection drug use	63
	Percutaneous needle stick	23
Sexual Exposure	*Receptive anal intercourse*	138
	Insertive anal intercourse	11
	Receptive penile-vaginal intercourse	8
	Insertive penile-vaginal intercourse	4
	Receptive oral sex	Low
	Insertive oral sex	Low
Vertical Transmission	*Mother-to-child transmission*	2260

Exposure risk in individuals not on therapy.

Table modified from P. Patel et al. (2014) AIDS [256]

1.3.1 Transcytosis and Mucosal Surfaces

Transcytosis occurs at polarised epithelial surfaces and is a method of trafficking across the membrane from the apical to basal layer. HIV-1 transcytosis involves viral entry into epithelial cells and transport across the membrane without direct infection of the cells [257]. It has also been demonstrated that FcRn may play a role in HIV-1 transcytosis [258]. Once through the epithelial barrier, the virus is then able to go on and infect sub-epithelial CD4+ target cells (Figure 1.10). Cell-free virus has been shown to transcytose through vaginal and genital epithelium [259], although the efficiency of viral transmission via this method is thought to be extremely poor [260].

Furthermore, transcytosis can occur through rectal and colonic tissue, with monoclonal antibodies to HIV-1 shown to block this mode of infection [261, 262].

1.3.2 Female Genital Tract

Male-to-female transmission of HIV-1 represents approximately 47% of global HIV-1 infection, rising to 57% in Sub-Saharan Africa (UNAIDS 2016 estimates: www.unaids.org). An undamaged epithelial layer forms a good, albeit not completely impenetrable, primary barrier against HIV-1 infection. However, differences in tissue structure throughout the female genital tract (Figure 1.11a), changes induced by hormones (both cyclical and contraceptive-based), age, sexual violence and the availability of HIV-1 target cells can all have an impact on the probability of an HIV-1 transmission event occurring.

1.3.2.1 Vagina and Ectocervix

The lower female genital tract comprises the vagina and ectocervix, tissues that are made up of a non-keratinised squamous epithelium, forming an excellent barrier against HIV-1 infection, providing it remains intact [245, 263]. However, disruption of the epithelial layers by other sexually transmitted diseases, inflammation or violent sexual activity increases the susceptibility of HIV-1 acquisition due to easier access to CD4+ target cells within the subepithelium. Within the epithelial layers, monocytic cells, DCs, LC and CD4+ T cells have all been found, with the potential of becoming infected or transmitting virions across the epithelium to the underlying subepithelium, where further CD4+ target cells reside [264, 265].

NHP models have demonstrated that, once in the subepithelium, a local foci of infection expands before the virus is transferred to the lymph nodes and systemic infection is established [43, 44]. While the exact processes involved in HIV-1 transmission across the epithelium remain to be determined, LC have been shown to transfer HIV-1 across the epithelial surfaces [264].

Fluctuations in some immune cell numbers exist throughout the menstrual cycle, with studies demonstrating that NK cell and lymphocyte numbers within the endometrium of the upper female genital tract can vary across the different stages of the menstrual cycle [266]. However, macrophage numbers within the vaginal tissue have been shown to remain constant throughout the menstrual cycle [267], while neutrophils have been found in higher numbers in the upper female genital tract, decreasing in quantity as the tract descends [267].

Evidence from both human and NHP studies have suggested that hormonal changes, either caused by the natural monthly cycle or through use of contraceptive hormones, can alter the thickness of ectocervical tissue, potentially increasing the opportunity for HIV/SIV to infect [268-270]. Furthermore, studies investigating the use of oral contraceptives have demonstrated that there is an increase in CCR5 expressing CD4+ T cells within the cervical tissue of women using hormonal contraception [271-273].

1.3.2.2 Transformational Zone and Endocervix

The transformational zone is a region within the cervix where ecto- and endocervical tissues meet. It is a potentially vulnerable region for HIV-1 transmission and has been shown to be the primary site of infection for other sexually transmitted diseases, such as human papilloma viruses (HPV) [274].

The endocervix, comprised of a single columnar epithelium varies in thickness throughout the hormonal cycle, from 10-30 μm [263]. While the endocervix represents a thinner barrier for the virus to traverse when compared to the ectocervix, this region of the female genital tract contains mucus, which has been shown to trap HIV-1 virions [275, 276]. As well as trapping virions, the mucus is also of an acidic pH and contains anti-microbial properties and immunoglobulins (both IgG and IgA), all of which help prevent infection [277].

The endocervix has more superficial CD4+ target cells when compared to the lower female genital tract, although fewer LC have been observed than within the ectocervix [264, 278]. Furthermore, an *ex vivo* study demonstrated that endocervical explants were more susceptible to HIV-1 infection when compared to ectocervical explants [265].

In addition, age has an impact on the thickness of the cervix. Menopause induces changes in the cervix, leading to thinning of the epithelial layers [279]. Furthermore, cervical ectopy, an outgrowth of endocervical columnar epithelium over the ectocervical surface, can increase the chance of transmission through microabrasions and trauma at this site. Cervical ectopy is more common in younger women, during pregnancy and in women taking oral contraceptives [280-282].

1.3.3 Male Genital Tract

Approximately 53% of those living with HIV-1 globally are male, acquired via female-to-male or male-to-male HIV-1 transmission (UNAIDS 2016 estimates: www.unaids.org). Once again, an intact epithelial layer forms the primary defence against HIV-1 in the male genital tract. However, differences

exist in the structural and immunological nature of the tissues of the foreskin, penile glans and urethra, which are potentially important factors to consider in terms of HIV-1 acquisition in males (Figure 1.11b).

1.3.3.1 Penile Foreskin and Glans

The penile foreskin (both inner and outer) and penile glans tissue are comprised of keratinised stratified squamous epithelium. There is some debate as to whether the inner foreskin is comprised of a thinner layer of keratinised cells when compared to the outer foreskin and glans tissues, with some studies providing evidence that it is thinner [283] and others stating that there is no statistical difference in the thickness [284-286]. Regardless, the keratinised epithelial layer represents a tough barrier to HIV-1 infection, providing it remains intact. As with the female genital tract, infection or injury leads to disruption of the epithelial layers and increases the rate of HIV-1 transmission. Individuals co-infected with other bacterial and viral sexually transmitted infections (STIs) are at an increased risk of HIV-1 infection [287, 288].

Once breached, CD4+ T cells, macrophages, LC and DCs, as well as cells expressing CCR5 and CXCR4 have been shown to reside within both the foreskin and glans tissues [77, 283]. LC are found within the superficial layers of the epithelia when compared to other target cells [283, 289], and are thought to be the first cells to come into contact with HIV-1, subsequently transferring the infection to the underlying CD4+ T cells, macrophages and DCs.

While the exact mechanism of HIV-1 transmission across penile tissues remains to be determined, is has been shown that HIV-1 acquisition can be reduced by up to 60% through the relatively simple approach of male circumcision [290-292], which involves removal of the foreskin, leaving behind

the glans penis as the outer surface. It has been suggested that the observed decrease in infection is simply due to the reduction in surface area for possible HIV-1 transmission events to occur [293]. However, further investigations into the mechanisms behind the reduction in HIV-1 acquisition in circumcised men have indicated that there may be a number of possible causes. These include a greater density of CD4+ T cells and Langerin-expressin LC within the inner foreskin compared to the outer foreskin [294], upregulation of inflammatory cytokines and co-receptor expression in the foreskin [295, 296], and removal of the foreskin leading to a 'drying out' of the penile glans tissue, toughening up the keratinised layer [297]. While male circumcision reduced the risk of HIV-1 acquisition during heterosexual intercourse, the evidence for it being an effective method of protection in homosexual HIV-1 transmission remains to be determined.

1.3.3.2 Penile Urethra

While circumcision reduces the rate of HIV-1 transmission by around 60%, there remains ~40% of incidences where transmissions still occur. A possible vulnerable site for HIV-1 acquisition is the penile urethra, which is formed of non-keratinised stratified squamous epithelium [298]. Although research into HIV-1 transmission via the penile urethra is limited, studies have shown that HIV-1 target cells are present within this tissue [298-300]. However, it is currently unclear as to whether there is an increased risk of HIV-1 acquisition via the penile urethra, with studies observing no significant difference in HIV-1 infection of *ex vivo* foreskin, penile glans, meatus and urethral explants by an R5-virus [301].

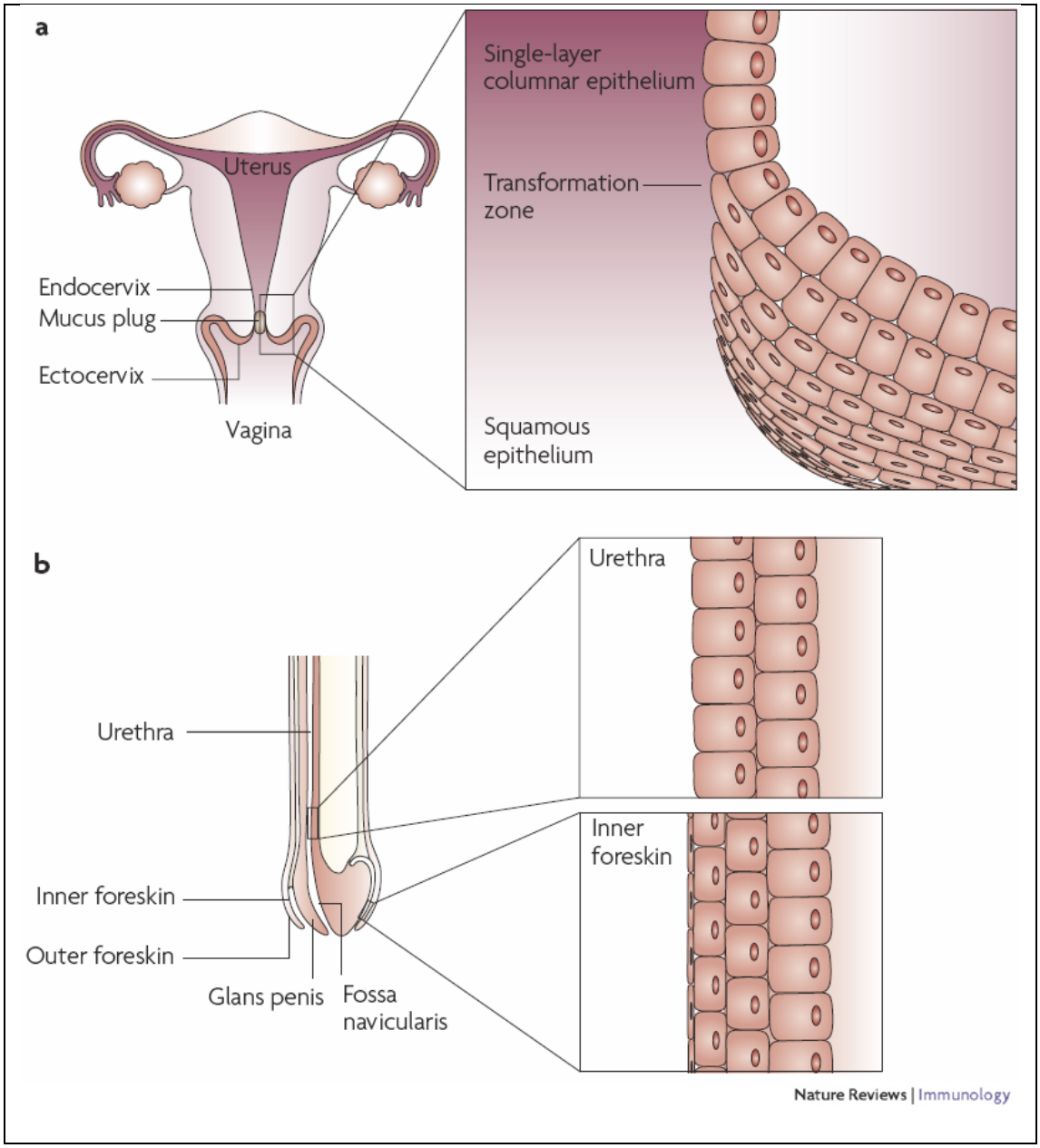


Figure 1.11 Illustrations of (A) the female reproductive tract and (B) the male reproductive tract.

Image taken from Hladik & McElrath (2008) [289].

1.3.4 Gastrointestinal Tract

The gastrointestinal tract has a crucial role in both HIV-1 transmission and subsequent pathogenesis. HIV-1 transmission can occur via the gastrointestinal tract through sexual practices or through mother-to-child transmission (MTCT) via the ingestion of virally infected blood or cervical mucus during birth or postnatal breast milk. Once HIV-1 has established a systemic

infection, the gastrointestinal tract is one of the primary areas affected due to massive CD4+ T cell depletion and loss of structural integrity [186].

1.3.4.1 Upper Gastrointestinal Tract

HIV-1 transmission can occur via the upper gastrointestinal tract through MTCT or oral-genital transmission during sexual intercourse. While *in utero* transmission of HIV-1 across the placenta can occur in approximately 7% of infected mothers [302], the majority of MTCT events occur during labour or through the ingestion of infected breast milk. Despite antiretroviral therapy being an effective preventative measure [303-306], MTCT via breast milk accounted for almost 200,000 new transmission events in 2015 (www.unaids.org).

Furthermore, oral-genital transmission of HIV-1 during sexual intercourse is a less frequent form of infection, but has been shown to occur [307, 308]. The increased risk of HIV-1 transmission via the oral route in infants is thought to be linked to differences in the stratified epithelial layers between infants and adults, with adults possessing a more highly stratified epithelium [309].

1.3.4.2 Lower Gastrointestinal Tract

More commonly, HIV-1 is transmitted via the lower gastrointestinal tract during anally-receptive sexual intercourse. Indeed, transmission events are thought to occur in 1.4% of each unprotected receptive anal intercourse act, which is approximately 18 times higher than for unprotected receptive vaginal intercourse acts [310, 311].

While the exact events involved in HIV-1 transmission across the lower gastrointestinal tract remain to be determined, is it known that the rectal epithelium is comprised of a single layer of columnar cells that is more vulnerable to injury and trauma than the epithelium of the female genital tract [312]. Moreover, there are numerous HIV-1 target cells residing within the rectal lamina propria, including CD4+CCR5+ T cells [313], as well as multiple antigen presenting cells, such as DCs and macrophages [314, 315]. In addition, it has been demonstrated that HIV-1 infection can prompt DC migration through the epithelial cells, where they efficiently capture virions and transfer them to the underlying CD4+ target cells [316]. Lastly, it has been shown that gp120 from some HIV-1 isolates can bind to the gut homing receptor integrin $\alpha_4\beta_7$ [45], increasing the susceptibility of gut homing CD4+ T cells for infection [49], with NHP studies demonstrating that the frequency of CD4+ $\alpha_4\beta_7$+ T cells correlated with susceptibility to rectal SIV infection [317]. Furthermore, a study in NHP suggested that administering anti-$\alpha_4\beta_7$ monoclonal antibodies to block these interactions with SIV led to reduction in mucosal viral transmission and protected the gut-associated lymphoid tissue from SIV infection [318].

From the acute stage of HIV-1 infection onwards, the gastrointestinal tract is subject to significant CD4+ T cell depletion, which is only partially rescued upon initiation of anti-retroviral therapy [319, 320]. In addition, there is loss of barrier integrity and increased microbial translocation across the lamina propria, which leads to the gastrointestinal symptoms of diarrhoea and malabsorption observed during the course of infection [321-323]. Furthermore, tissue-resident macrophages of the gastrointestinal system are one of the key

cells involved in the development and sustainment of the HIV-1 viral reservoir [324].

1.4 Prevention Strategies, Treatments and Vaccines

While condoms remain the most effective form of prevention against sexually transmitted HIV-1, studies have highlighted the importance of education and alternative strategies. Male circumcision has been shown to reduce the incidence of HIV-1 transmission by ~60% [291, 292]. Furthermore, behavioural counselling and the encouragement of partner number reduction have been shown to lower the incidence of HIV-1 transmission [325, 326]. Importantly, MTCT and infection via intravenous drug use or blood transfusions cannot be prevented using barrier methods. The use of HIV-1 drugs and treatments remains vital for the prevention of these forms of transmission. The incidence of MTCT has been dramatically reduced due to increased testing of all pregnant women (at least within the UK), leading to antiretroviral therapy during pregnancy, the use of caesarean section method of delivery and the advice not to breastfeed. However, in developing countries, prevention of breastfeeding increases the risk of contracting other infectious diseases, which pose a greater threat to the survival of an infant than HIV-1 infection. The World Health Organisation (WHO) therefore recommends that breastfeeding should continue within these nations [327].

1.4.1 Current Prevention Drugs and Treatments

Research to discover a treatment for HIV-1 began soon after the virus was identified as being the cause of AIDS. The first drug to be identified and given Food and Drug Administration (FDA) approval for general use in the USA in 1987 was Azidothymidine (AZT). Originally a drug developed to treat cancer, AZT works by inhibiting reverse transcriptase [328-330]. However, it was quickly observed that HIV-1 could mutate to develop resistance to AZT on its own [331]. Modern HIV-1 prevention strategies have evolved to use highly active antiretroviral therapy (HAART), usually combining at least three different HIV-1 drugs to different targets, given simultaneously to prevent HIV-1 developing resistance and to confer a fitness cost to the virus (Figure 1.12). In 2015, of the 36.7 million people infected with HIV-1 globally, around 17 million had access to antiretroviral drugs (www.unaids.org).

1.4.1.1 Attachment and Fusion Inhibitors

These drugs function by interrupting the binding of HIV-1 to either the primary receptor (CD4) or the co-receptors (CCR5/CXCR4) or by preventing the fusion and integration of gp41 into the cellular membrane. Examples include Maraviroc (a CCR5 inhibitor) and Enfuvirtide (also known as T-20; blocks gp41 to prevent viral entry) [332].

1.4.1.2 Nucleoside/non-nucleoside Reverse Transcriptase Inhibitors (NRTI/NNRTI)

Both NRTIs and NNRTIs work by blocking HIV-1 reverse transcription enzymes needed to synthesise viral DNA. Examples include AZT, Emtricitabine (NRTI) and Etravirine (NNRTI) [332].

1.4.1.3 Integrase Inhibitors

Integrase inhibitors (also known as integrase strand transfer inhibitors) block the activity of integrase, a viral enzyme used to insert viral DNA into the host DNA. Examples include Dolutegravir and Elvitegravir [332].

1.4.1.4 Protease Inhibitors

These drugs prevent the release of infectious viral particles by blocking the viral protease responsible for viral cleavage of the Gag polyprotein. Examples include Saquinavir and Ritonavir [332].

1.4.1.5 Combination Therapy

Current HIV-1 prevention strategies and treatments use highly active antiretroviral therapy (HAART). These drugs are most commonly provided as combined pills to improve ease of treatment and promote adherence to the treatment regime.

1.4.1.6 Pre-Exposure Prophylaxis (PREP)

As well as being used as a treatment for those already infected with HIV-1, ART can also be utilised as a pre-exposure prophylaxis (PREP) preventative intervention to block infection. Several clinical trials have demonstrated that the use of Truvada, a combination therapy comprising Tenofovir and Emtricitabine, reduces the chance of HIV-1 transmission in high-risk individuals by up to 99%, providing there is good adherence to the drug [333-336].

1.4.1.7 Post-Exposure Prophylaxis (PEP)

Post-exposure prophylaxis (PEP) can be used to prevent HIV-1 infection after a potential exposure event that may have occurred either through high-risk sexual activity or during occupational exposure, such as needle-stick injury. PEP

must be administered within 72 hours of a potential exposure and involves taking HAART over the course of a month [337].

1.4.1.8 Microbicides

The use of ARTs topically as microbicides to prevent the sexual transmission of HIV-1 has been extensively researched. The CAPRISA 004 clinical trial in South Africa demonstrated a reduction in HIV-1 acquisition of 39% in women who applied 1% tenofovir gel vaginally prior to sexual intercourse [338]. More recently, The Ring Study/ASPIRE demonstrated that the use of a monthly vaginal ring device designed to continually release dapivirine prevented HIV-1 infection, although there were differences observed in the efficacy that were dependent on age and adherence [339].

1.4.1.9 Treatment as Prevention

The use of HAART in HIV-1 positive patients can reduce viral loads to undetectable levels, meaning that these individuals are significantly less likely to transmit virus. The use of HAART to control viral loads is so effective that it is now a potent tool for preventing the onward transmission of the virus in serodiscordant couples and during MTCT [340-342].

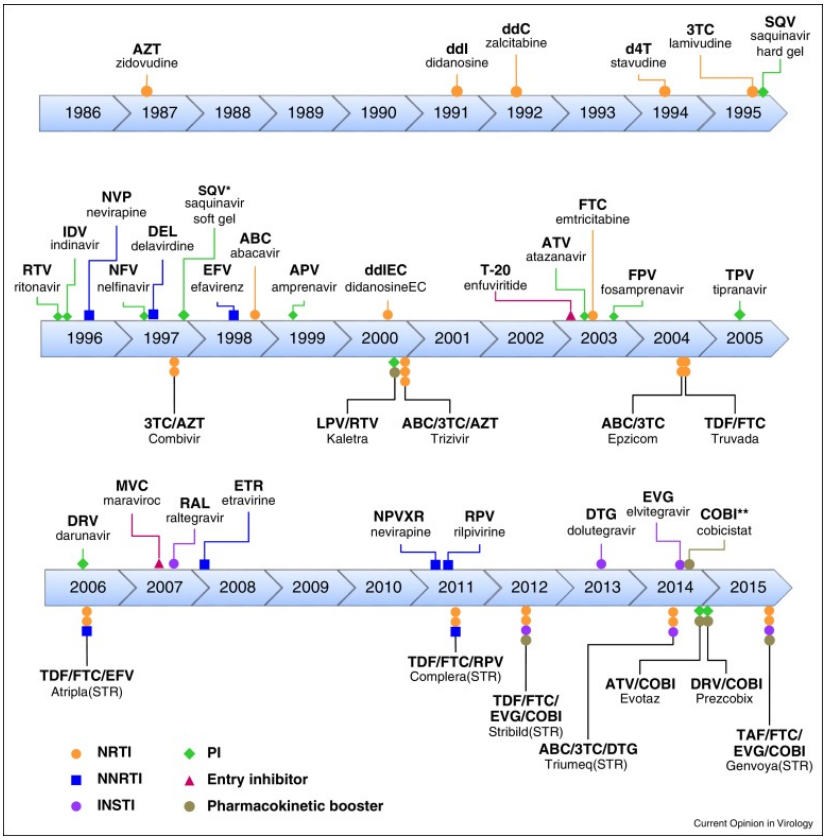


Figure 1.12 FDA-approved individual antiretroviral drugs and drug combinations

Taken from T. Cihlar & M. Fordyce (2016) Current Opinion in Virology [343].

1.4.2 Vaccines

Despite the development of numerous HIV-1 treatments and prevention strategies, the epidemic remains a huge global burden, particularly in sub-Saharan Africa. In 2015, it was estimated that there were 2.1 million individuals newly infected with HIV-1 and 1.1 million AIDS-related deaths (www.unaids.org). It is generally accepted that an effective vaccine is the only way to reverse the HIV-1 epidemic.

1.4.2.1 A Brief History of Vaccines

It was Edward Jenner who first tested the theory of vaccination in 1796 after he observed that milkmaids exposed to cowpox, a relatively mild disease, appeared to be immune to the more virulent smallpox. To test his hypothesis

that cowpox was conferring immunity to smallpox, he enlisted the eight-year-old son of one of his gardeners. He obtained infectious material from cowpox pustules on the hands of one of the milkmaids and used it to infect the boy, who became symptomatic for the disease. Once the symptoms had resolved, Jenner re-challenged the boy with smallpox on two separate occasions, but the boy remained smallpox-free [344]. He went on to repeat this experiment in a further 23 participants, before publishing his findings in a paper entitled *'An Enquiry into the Causes and Effects of the Variolae Vaccinae'* in 1798 [345].

Since these very early days, vaccinology has progressed, with many infectious diseases now controlled through vaccination programmes. Indeed, in 1980, after an intensive worldwide vaccination effort, smallpox became the first infectious disease to be declared globally eradicated by the World Health Assembly [346].

1.4.2.2 Forms of Vaccines

Almost all vaccines function through inducing antibody-driven humoral immunity, with the aim of conferring immunological memory against the pathogenic form of the disease. Different types of vaccine exist, including live-attenuated and inactivated vaccines, whereby patients are vaccinated with live pathogen that has been altered to make it less pathogenic or a killed version of the pathogen, respectively. Protein subunit vaccines induce an immune response through the administration of fragments of the pathogen, whereas DNA vaccines use DNA from the infectious agent. To boost the immune response during vaccination recombinant vector vaccines have been developed, which combine the DNA of the desired immunogen packaged within the vector of a different immunogen [347]. Finally, vaccine regimes have been developed to enhance the

immune response to a vaccine, for example, prime-boost vaccine regimes might employ an initial prime with a DNA vaccination, followed some weeks later with a recombinant vector boost vaccination [348].

1.4.3 HIV-1 Vaccines

To date, there have been numerous HIV-1 vaccine trials that have got as far as phase I clinical trials in humans, although few have gone further than this stage, usually due to lack of sufficient immunogenicity.

The VaxGen trials, VAX003 and VAX004, enrolled participants from Thailand and the USA and Europe, respectively, and were the first phase III HIV-1 vaccine trials to be conducted. It was hypothesised that the vaccines would induce neutralising antibodies to HIV-1 capable of clearing infection before it became established. However, the results revealed the vaccines demonstrated no efficacy in preventing HIV-1 infection or lowering viral load upon seroconversion [349, 350]. Due to the disappointing results, subsequent vaccine trials moved to elicit a T cell response. However, the phase IIb STEP/Phambili vaccine trials in South Africa and the USA demonstrated a possible increase in HIV-1 acquisition in vaccine participants, potentially due to CD4+ T cell activation [351, 352]. The most successful vaccine trial to date is the phase III RV144 trial, conducted in Thailand. This trial demonstrated 31.2% efficacy, which correlated to the production of non-neutralising IgG, ADCC-inducing antibodies to the V1V2 region of HIV-1 Env, in the absence of IgA to the same region of Env [353-358]. The general consensus for vaccine design is now that a successful HIV-1 vaccine should be able to induce both B- and T cell immunity.

1.4.3.1 VaxGen Trials

1.4.3.1.1 VAX003

Vax003 was a phase III randomised, double-blind, placebo-controlled vaccine trial that took place in Thailand between 1999 and 2000. The study enrolled 2546 HIV-1 negative participants who were intravenous drug users (IDUs) at high-risk of HIV-1 infection. The participants were vaccinated intramuscularly over seven time points with alum-adjuvanted AIDSVAX B/E, containing two recombinant gp120 proteins isolated from subtype B and E HIV-1 strains. Reduction in HIV-1 infection rates were the primary outcome for vaccine efficacy, with secondary endpoints being safety and delayed disease progression. In addition, the participant samples were tested for gp120-specific antibody responses. The results demonstrated that the vaccine elicited no protective efficacy, with 8.4% of the vaccine group seroconverting versus 8.3% of the placebo-control group. Furthermore, there was no significant difference in disease progression or gp120-specific antibody production between the vaccinated and placebo groups [350]. While the vaccine was well-tolerated and deemed safe, the lack of efficacy meant that it was discontinued as a stand-alone vaccine.

1.4.3.1.2 VAX004

Vax004 was a phase III randomised, double-blind, placebo-controlled vaccine trial that took place in the USA and Europe in 2003, before the results of the Vax003 trial became available. The study design and vaccination regimen were the same as for Vax003, this time enrolling men who have sex with men (MSM) and high-risk female volunteers to receive the alum-adjuvanted AIDSVAX

B/B vaccine. The vaccine contained two recombinant gp120 envelope antigens derived from two separate subtype B HIV-1 strains. Once again, primary outcome was to determine vaccine efficacy through decreased infection rates, with secondary outcomes of safety and delayed disease progression. The results demonstrated no efficacy, with 6.7% of the vaccinees seroconverting versus 7.0% of the placebo group. Furthermore, the vaccine demonstrated no significant ability to reduce viral load post-infection [349]. Subsequent analysis of the trial samples demonstrated higher CD8+ T cell responses in vaccinees who seroconverted [359]. Once again, the vaccine was found to be safe and well-tolerated, however, it too was discontinued as a stand-alone vaccine due to lack of efficacy.

1.4.3.2 The STEP/Phambili Trial

The STEP/Phambili trials were multi-centre, double-blind, randomised, placebo-controlled, test-of-concept phase IIb studies using an Ad5 vector vaccine with genes for HIV-1 *gag, pol, nef* inserted, designed to induce T cell responses. HIV-1 negative male and female participants at high risk of HIV-1 infection were recruited at sites across North America, South America, Australia, the Caribbean (STEP trial) and South Africa (Phambili Trial) [351, 352]. The studies divided the participants into two groups; those with Ad5 antibody titres <200 (seronegative) and those with Ad5 antibody titres >200 (seropositive). The main objectives of both studies were to show safety, tolerability and efficacy (defined as a decrease in HIV-1 infection in the vaccinated group over the placebo group or decrease in early viral loads) [360]. However, in 2007, the STEP trial was halted early due to safety concerns when interim analysis showed increased seroconversion rates in the Ad5 seropositive, vaccinated group over the other

groups. In 2008, the Phambili Trial was also halted due to concerns raised over the results of the STEP trial. While the increase in HIV-1 seroconversion never reached statistical significance, it was always higher in the Ad5 seropositive groups. Subsequent follow-up analyses of the vaccine participants have continued to demonstrate a higher rate of HIV-1 infection in uncircumcised Ad5 seropositive males, while the vaccine conferred no protection in female participants [361-363].

Sieve analysis of these trials demonstrated that, while there may be some vaccine-induced immune pressure on the founder virus, no specific CD8+ T cell response could be identified [364]. Further investigations failed to elucidate the reasons behind the increase in HIV-1 acquisition in the uncircumcised Ad5 seropositive participants. Some studies claim that the pre-existing immunity to Ad5 was not responsible for the increase susceptibility to HIV-1 [365, 366]. However, other investigations have shown that the Ad5 vector induced activation of CD4+ T cells [367], potentially of gut-homing phenotype [368], suggesting that an influx of activated CD4+ T cells may have provided an enhanced environment for HIV-1 infection. Whether pre-existing immunity to adenovirus was to blame for the increased infection rates, the HIV-1 vaccine field has moved away from using this viral vector for the time being. However, the use of Chimpanzee adenoviruses as vectors has demonstrated some promising results, highlighting potential for their use in future HIV-1 vaccine trials [369, 370].

1.4.3.3 RV144 Vaccine Trial

The phase III RV144 vaccine trial was a randomised, multicentre, double blind, placebo controlled trial carried out in community risk volunteers in Thailand in 2009 and is the most promising HIV-1 vaccine trail to date [353].

The prime-boost vaccine regime was comprised of 4 primes with ALVAC-HIV (canarypox vector expressing HIV *gag, env* and *pol* genes) followed by two boosts with AIDSVAX B/E, which was a genetically engineered HIV-1 gp120 Env protein. Use of these vaccine components caused some controversy and criticism from vaccine experts at the time because, in two separate vaccine trials, the individual vaccines had demonstrated little immunogenicity and it was thought futile by some to begin a new vaccine trial using ALVAC-HIV and AIDSVAX B/E [349, 350, 371, 372].

However, the vaccine trial went ahead, enrolling 16402 healthy male and female participants at low risk of HIV-1 infection. Results showed an efficacy of 31.2% in vaccinated individuals, which was determined by examining the 125 participants of the trial who became infected with HIV-1 after 42 months of follow-up. Of these 125 participants, 74 were found to be in the placebo group, whereas 51 were in the vaccinated group [353]. Once infected, the course of disease was not altered by vaccination, with no difference observed in viral set-point, post-infection CD4 counts or time to AIDS between the vaccinated and placebo groups [353, 373].

The initial published results showed that the vaccine produced weak CD8+ T cells responses [353], with subsequent analysis revealing a lack of bnAb production [374]. However, extensive correlates of protection analysis have since been performed that demonstrate a clear positive correlation between

levels of non-neutralising IgG antibodies against the gp120 variable regions V1V2 of the envelope glycoprotein structure, with ADCC activity and a reduced risk of infection. This protection was abrogated in the presence of IgA to the same region of gp120 [354, 355, 358, 375].

Further analysis of the antibodies induced during the trial revealed IgG3 antibodies to the V1V2 region with high ADCC activity appeared early after vaccination, but waned over time, correlating with the protection observed [134]. Furthermore, polymorphisms within CD32C, which can impact Fc-binding and effector functions, have been shown to correlate with protection in some participants of the trial [135]. Additionally, investigations have also highlighted potential correlations between class I HLA alleles and vaccine efficacy, with individuals with HLA A*02 demonstrating greater vaccine efficacy than individuals with other HLA alleles [376].

There was a lack of mucosal sampling within the RV144 trial, so any mucosally-derived correlates of protection could not be assessed. However, subsequent trials in NHP and follow-up studies in human participants will hopefully provide further insight into the immune responses key to successful vaccine-induced immunity.

In many ways, the results from the RV144 trial are the basis for this research and work by other groups. Data from the RV144 trial have strengthened the idea that nnAb and ADCC should not be overlooked when considering vaccine development. However, the results leave many questions unanswered, including whether nnAb and ADCC are true correlates of protection or mechanistic correlates, linked to alternative modes of protection.

1.5 Overall Aims

The overarching hypothesis of this thesis is that the protective efficacy of HIV-1 antibodies is likely dependent upon their functional activity in mucosal tissue that may not be predicted by conventional neutralisation. The thesis aims to assess functional activity in a range of tissue models thought to mimic the initial steps required to establish infection. Critical to these models is the initial characterisation of target cells for HIV-1 infection and effector cells involved in antibody function.

1.5.1 Aims

1.5.1.1 Characterise the expression profiles of Fc Receptors in mucosal tissue, specifically colorectal, penile glans and ectocervical.

As antibody function is likely critically dependent upon FcR function (particularly for nnAb), FcR expression levels, effector cell location and frequency in the different tissues will be assessed using flow cytometry panels and confocal microscopy.

1.5.1.2 Determine the role of antibody Fc-mediated antiviral activity in different tissue models.

The role of antibody Fc-mediated antiviral activity will be assessed in different tissue and cellular models designed to mimic the first events required to establish mucosal infection. To this end a panel of nAb and nnAb, including

those associated with protection in the RV144 vaccine trial, will be assessed for antiviral activity.

1.5.1.3 Determine the role of infiltrating peripheral cells on HIV-1 infection in the presence of neutralising and non-neutralising antibodies.

The role of whole blood on the activity of nAb and nnAb at reducing HIV-1 infected CD4+ cells will be assessed. Further to this, the ability of nAb and nnAb to bind to HIV-1 infected CD4+ cells will be investigated to determine whether there is a correlation between the reduction in infection observed by the HIV-1 specific antibody and the ability of the antibody to bind to infected cells.

1.5.2 Outline

Chapter 2 outlines the materials and methods used throughout this thesis.

Chapter 3 investigates the expression of FcR (CD16, CD32, CD64 and CD89) and effector cell phenotypes (CD14+ monocytic cells, myeloid DCs and NK cells) in blood, penile glans, ectocervical and colorectal tissue using multiparametric flow cytometry panels and deconvolution microscopy.

Chapter 4 investigates the function of neutralising and non-neutralising antibodies at reducing HIV-1 infection in cellular and mucosal tissue models designed to mimic the early events in HIV-1 transmission.

Chapter 5 investigates a whole blood model to compare the reduction of HIV-1 infection by neutralising and non-neutralising antibodies to immunoglobulin binding to infected cells.

Chapter 6 summarises and concludes the results of this thesis and discusses limitations and future research.

CHAPTER 2 – MATERIALS AND METHODS

2 Materials and Methods

2.1 Reagents

2.1.1 Materials

Table 2.1 Commercially available reagents

Material	Supplier	Catalogue number
0.2 μm syringe filters	Sartorius Stedim	16354GUK
15 mL Centrifuge Tubes	Sigma	T1943
50 mL Centrifuge Tubes	Sigma	T2318
60 mm petri dishes	Corning	391-1917
70 μm cell strainers	BD Biosciences	352350
White 96-well, flat-bottom, high-bind plates	Corning	CC705
AALTO p24 ELISA Coating Antibody, Polyclonal	AALTO Bio Reagents Ltd	D7320
AALTO p24 ELISA Secondary Conjugate Antibody, Polyclonal	AALTO Bio Reagents Ltd	BC 1071-AP
ACK Lysing Buffer	Lonza	10-548E
AIM-V medium	Thermo Fisher Scientific	12055091
Amphotericin B (Fungizone)	GE Hyclone	11526481
Anti-Mouse Ig, κ/Negative Control Compensation Beads	BD Biosciences	552843
Anti-Rat Ig, κ/Negative Control Compensation Beads	BD Biosciences	552844
ArC™ Amine Reactive Compensation Bead Kit	Molecular Probes	A-10346
CytoFix/CytoPerm kit	BD Biosciences	554714
Dimethyl Sulfoxide (DMSO)	Sigma	D2650
DNase I	New England Biolabs	M0303S
Dried whole milk powder	AppliChem. Inc.	A0830
Dulbecco's Modified Eagles Medium (DMEM)	Sigma	D6546
Dynabead CD4 Positive Isolation Kit	Invitrogen	11331D
DynaMag™ -15	Invitrogen	12301D
ELISA-light Immunoassay System with CSPD and Sapphire-II Substrate/Enhancer Solution	ThermoFisher Scientific	T1023
Empigen BB Detergent	Sigma	45165
FACS tubes	BD Biosciences	352063
Flat-bottom culture plates, 96-well	Corning	353072
Freestyle media	Gibco	12338
Gentamicin	Sigma	G7513-100 mL
Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF)	R & D Systems	215-GM
Heat-inactivated Foetal Calf Serum	Gibco	10500

Histopaque	Sigma	H8889-500 mL
Lysed HIV-1 (MN) CL-4 H9 ELISA Standard	Frederick National Laboratory for Cancer Research	N/A
Human Immunodeficiency Virus-1 $_{BaL}$	NIH AIDS Reagent Program	510
Human HIV-1 Antibody 2F5	Polymun Scientific	AB001
Human HIV-1 Antibody 2G12	Polymun Scientific	AB002
Human HIV-1 Antibody 4B3	Polymun Scientific	AB012
Human HIV-1 Antibody 4E10	Polymun Scientific	AB004
Human HIV-1 Antibody b12	Polymun Scientific	AB011
Human HIV-1 Antibody F240	Polymun Scientific	AB017
Human HIV-1 Antibody PG16	Polymun Scientific	AB016
Human HIV-1 Antibody PG9	Polymun Scientific	AB015
Hyaluronidase	Sigma	H3884-50 mg
Interleukin-2	R & D Systems	202-IL
Interleukin-4	R & D Systems	204-IL
L-Glutamine	Sigma	G7513-100 mL
Leucoagglutinin (PHA-L), lyophilized powder	Sigma	L2769
Liberase DH	Roche	05401054001
Liberase DL	Roche	05401160001
Liberase TH	Roche	05401135001
Liberase TL	Roche	05401020001
Liberase TM	Roche	05401119001
LIVE/DEAD Fixable Aqua Dead Stain Kit	Molecular Probes	L34956
Luciferase	Promega	E4530
Mouse anti-human CD11c A700, B-ly6	BD Biosciences	561352
Mouse anti-human CD123 PECy5, 9F5	BD Biosciences	561009
Mouse anti-human CD14 Qdot 605, TüK4	Molecular Probes	Q10013
Mouse anti-human CD15 BV650, W6D3	Biolegend	323034
Mouse anti-human CD16 Pacific Orange, 3G8	Molecular Probes	MHCD1630
Mouse anti-human CD16 PECy7, 3G8	Biolegend	302016
Mouse anti-human CD19 FITC, HIB19	BD Biosciences	560994
Mouse anti-human CD3 V450, UCHT1	BD Biosciences	560365
Mouse anti-human CD3 PECy7, OKT3	Biolegend	317334
Goat anti-human α-IgG A647, Polyclonal	Invitrogen	A-21445
Mouse anti-human CD32 APC, FLI8.26	BD Biosciences	559769
Mouse anti-human CD4 PE, RPA-T4	BD Biosciences	555347
Mouse anti-human CD4 PECy7, RPA-T4	Biolegend	300512
Mouse anti-human CD45 A700, HI30	Biolegend	304024
Mouse anti-human CD56 PECy5, HCD56	Biolegend	318308

Mouse anti-human CD64 APC H7, 10.1	BD Biosciences	561190
Mouse anti-human CD66b FITC, G10F5	Biolegend	305104
Mouse anti-human CD8 APC, RPA-T8	BD Biosciences	555369
Mouse anti-human CD89 PE, A59	BD Biosciences	555686
Mouse anti-human p24 FITC, KC57	Beckman Coulter	6604665
Penicillin/Streptomycin	Gibco	15140-122
Phosphate-buffered Salt Solution (PBS)	Sigma	P5368
PM-1 cell line	NIH AIDS Reagent Program	3038
RETROtek HIV-1 p24 Antigen ELISA Kit	ZeptoMetrix Corporation	0801200
RPMI medium	Sigma	R0883
Sheep Serum	Sigma	S2263-100 mL
T-175 flasks	Corning	431466
T-25 flasks	Corning	431463
T-75 flasks	Corning	431464
Tris-buffered salt solution (TBS)	Sigma	T5912
Trypsin-EDTA	Sigma	T4049
Tween-20	ThermoFisher Scientific	337-500
TZM-bl cell line	NIH AIDS Reagent Program	8192
U-bottom culture plates, 96-well	Corning	734-0027

2.2 Media and Other Solutions

2.2.1 Complete RPMI

Roswell Park Memorial Institute medium (RPMI) supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), 2 mM L-glutamine and 10% heat-inactivated Foetal Bovine Serum (FBS).

2.2.2 Complete DMEM

Dulbecco's Modified Eagle's Medium (DMEM) supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), 2 mM L-glutamine and 10% heat-inactivated Foetal Bovine Serum (FBS).

2.2.3 Colorectal Tissue Culture Medium

Dulbecco's Modified Eagle's Medium (DMEM) supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), 2 mM L-glutamine, 50 μg/mL gentamicin, 1 μg/mL fungizone and 10% heat-inactivated Foetal Bovine Serum (FBS).

2.2.4 Penile and Ectocervical Tissue Digestion Cocktail

Serum-free complete RPMI supplemented with 12.5 μg/mL Liberase TL, 25 μg/mL Hyaluronidase, 200 μg/mL DNase.

2.2.5 Colorectal Tissue Digestion Cocktail

Serum-free complete RPMI supplemented with 12.5 μg/mL Liberase DL, 25 μg/mL Hyaluronidase, 200 μg/mL DNase.

2.2.6 CytoFix/CytoPerm Wash Buffer

10x buffer was diluted 1:10 with dH_2O.

2.2.7 0.05% PBS/Tween (PBST) Buffer

500 μL Tween-20 added to 1 L PBS

2.2.8 TBS Buffer

10x buffer was diluted 1:10 with dH_2O.

2.2.9 0.1% Empigen in TBS

175 μL 35% Empigen was added to 50 mL 1x TBS.

2.2.10 AALTO p24 ELISA conjugate

For each full 96-well plate, 10 mL conjugate was prepared:

8 mL 1x TBS

2 mL Sheep Serum

50 μL 10% PBST

1.25 μL ELISA p24 secondary conjugate

2.3 Cell Lines

2.3.1 TZM-bl Cells

TZM-bl indicator cells, previously described as JC53-bl (clone 13), are derived from a HeLa cell line and stably express CD4, CXCR4 and CCR5. The TZM-bl cell line has been genetically modified to express *Escherichia coli* β-galactosidase and firefly luciferase genes under the transcriptional control of the HIV LTR. TZM-bl cells are highly susceptible to HIV infection and introduction of the indictor genes allows for easy quantitative analysis of HIV infection [377, 378]. TZM-bl cells are an adherent cell line and were cultured in complete DMEM medium. Every 3-4 days the TZM-bl cells were washed once with Phosphate Buffered Saline (PBS) and incubated with 1x Trypsin-EDTA for 5 mins at 37°C to allow detachment. Post-detachment, the cells were washed 1x with complete DMEM medium and passaged 1 in 10. The cells were cultured in a 95% humidified incubator with 5% CO_2 at 37°C.

2.3.2 PM-1 T cells

PM-1 cells are CD4+ T cells clonally derived from a HUT-78 cell line. The cells are permissive for growth of macrophage and T cell-specific HIV-1 [379]. PM-1 cells are grown in suspension in complete RPMI. Every 3-4 days the PM-1 cells were washed once with complete RPMI medium and passaged 1 in 10. The cells were cultured in a 95% humidified incubator with 5% CO_2 at 37°C.

2.4 Primary Cells

2.4.1 Peripheral Blood Mononuclear Cells (PBMC)

Blood was freshly isolated from HIV-negative donors and processed within 6 h. Blood was layered on to 20 mL room temperature histopaque and centrifuged for 30 minutes at 400x g. The mononuclear layer was recovered and washed once in 50 mL complete RPMI followed by centrifugation for 5 minutes at 400x g. Red blood cells were lysed with 1-5 mL ACK lysis buffer for 5 minutes and washed with complete RPMI before being resuspended in 10 mL complete RPMI and counted.

2.4.2 Leucocyte Cones

Leucocyte cones were obtained from NHS Blood and Transplant Centre, Colindale, London, UK. Cones were diluted 1:1 with PBS and layered on to 20 mL room temperature histopaque. Tubes then underwent the exact procedure as described in 2.4.1.

2.4.3 Red Blood Cell-lysed Whole Blood

Whole blood was isolated from HIV-1 negative donors mixed 1:1 with 1x RBC lysis buffer for 5 minutes at room temperature in the dark. Cells were washed twice with complete RPMI and the pellet was resuspended in 5 mL ACK lysis buffer for 5 minutes to remove any residual RBC. Cells were washed twice in PBS with centrifugation prepared for flow cytometry staining, as described in 2.8.

2.4.4 Monocyte-derived Macrophages (MDM)

Freshly isolated PBMC were washed and resuspended at $3x10^6$ cells/mL in serum free AIM-V medium containing 20 ng/mL GM-CSF. Cells were seeded at $3x10^5$ cells in 100 μL in flat-bottomed high binding 96-well plates and incubated at 37°C (5% CO_2). After 3-4 days, fresh serum free AIM-V media supplemented with 20 ng/mL GM-CSF was added and the cells were cultured for a total of 7 days.

2.4.5 Monocyte-derived Dendritic Cells (MDDC)

Freshly isolated PBMC were used to separate mononuclear cells by CD14 positive selection using an AutoMacs separation system.

20 μL of CD14 MicroBeads were added per 10^7 cells and incubated for 20 minutes at 4°C. Cells were washed once with AutoMacs running buffer by centrifugation for 10 minutes at 400x g and resuspended in 2 mL of AutoMacs running buffer for magnetic separation.

CD14 positive cells were resuspended in 40 mL complete RPMI supplemented with 30 ng/mL IL-4 and 25 ng/mL GM-CSF and cultured for 3-4 days at 37°C (5% CO_2). Media was replaced after 3-4 days with fresh RPMI complete medium supplemented with 30 ng/mL IL-4 and 25 ng/mL GM-CSF and incubated for a further 3 days at 37°C (5% CO_2).

2.5 Human Tissue

2.5.1 Ethics Statement

Written informed consent was obtained from all donors. All tissues were collected under protocols approved by the Imperial College NHS Trust Tissue

Bank and the National Research Ethics Committee in accordance with the Human Tissue Act 2004. Approval for this project was granted by the Imperial College Healthcare Tissue Bank, under their HTA research licence, and ethics thus conveyed through this process by the Multi Research Ethics Committee (MREC), Wales.

2.5.2 Ectocervical Tissue

Ectocervical tissue was acquired from women undergoing planned therapeutic hysterectomy at St Mary's Hospital (London, United Kingdom).

2.5.3 Penile Tissue

Penile glans tissue was acquired from men undergoing elective gender reassignment surgery at Charing Cross Hospital (London, United Kingdom).

2.5.4 Colorectal Tissue

Surgically resected specimens of colorectal tissue were collected at St Mary's Hospital (London, United Kingdom) from patients undergoing rectocele repair and colectomy for colorectal cancer. Only healthy tissue obtained 10 to 15 cm away from any tumour was employed.

2.6 Virus Culture

2.6.1 HIV-1_{BaL}

HIV-1_{BaL} virus is a macrophage and monocyte-tropic clade B laboratory isolate originally obtained from human infant lung tissue [380].

2.6.1.1 Growth in PBMC

Pooled blood from three leucocyte cones was incubated with 3 mL RosetteSep CD8 depletion cocktail for 20 minutes at room temperature. After the incubation, the PBMC were isolated using the exact procedure detailed in 2.4.1. After the PBMC isolation, 4 x 10^7 cells were stimulated in 40 mL volumes in three separate T-75 flasks for 72 h with 0.4 μg/mL OKT3 + 10 U/mL IL-2, 5 μg/mL PHA-L +10 U/mL IL-2 and 0.5 μg/mL PHA-L + 10 U/mL IL-2. After 72 h the cells from the three stimulations were pooled and resuspended to 1x10^6 cells/mL ready for infection with HIV-$1_{BaL.}$ PBMC were added to HIV-1_{BaL} at a 2:1 ratio and cultured for 3-4 days in a 95% humidified incubator with 5% CO_2 at 37°C. After this, the supernatant was harvested by centrifugation and fresh, uninfected PBMC were added to the infected PBMC at a ratio of 1:1. Every 3-4 days, the supernatant was harvested and the cell culture was doubled using uninfected PBMC. This process continued until sufficient virus had been grown.

2.6.1.2 Growth in PM-1 T cells

PM-1 T cells were resuspended at 1x10^6 cells/mL in 1 mL complete RPMI and 1 mL HIV-1_{BaL} was added in a 24-well culture plate. Cells and virus were incubated in a 95% humidified incubator with 5% CO_2 at 37°C for 3-4 days. Supernatant was harvested and fresh, uninfected PM-1 T cells were added to the infected PM-1 T cells at a ratio of 1:1. The virus supernatant was harvested and the culture volume was doubled every 3-4 days until sufficient virus had been grown.

2.7 Human Tissue Digestion

2.7.1 Penile and Ectocervical Tissue Digestion

Tissue was cut into 3 mm^3 explant-sized pieces. A maximum of 5 of these pieces were further diced using a scalpel blade and placed into 1 mL enzyme digestion cocktail for 2 x 1.5 h at 37°C with 1200 rpm shaking. After each 1.5 h enzymatic digestion, the tissue was further mechanically disrupted and isolated cells were washed with complete RPMI, collected and passed through a 70 μm nylon filter. Cells were stained with the flow cytometry panels (Tables 2.2 and 2.3) immediately after isolation.

2.7.2 Colorectal Tissue Digestion

Tissue was cut into 3 mm^3 explant-sized pieces. A maximum of 5 of these pieces were further diced using a scalpel blade and then placed into 1 mL of Liberase enzyme digestion cocktail for 1 h at 37°C with 1200 rpm shaking. After enzymatic digestion, the tissue was further mechanically disrupted and isolated cells were washed with complete RPMI, collected and passed through a 70 μm nylon filter. Cells were stained with the flow cytometry panels (Tables 2.2 and 2.3) immediately after isolation.

2.8 Flow Cytometry

2.8.1 Viability Staining

Aqua viability dye was diluted 1:400 in PBS. After isolation from tissue or blood, cells were washed once with PBS by centrifugation and incubated with

100 μL Aqua viability dye at room temperature for 20 minutes in the dark. After incubation, cells were washed twice with PBS using centrifugation.

2.8.2 Surface Staining

Post-viability dye staining, cells were incubated for 20 minutes at RT in the dark with 50 μL final volume of one the surface staining panels listed below in Tables 2.2 and 2.3 for Fc receptor phenotyping. All antibodies were titrated to ensure optimum staining levels. After incubation, cells were washed twice with PBS using centrifugation. Cells were fixed with 3% paraformaldehyde and placed at 4°C in the dark until acquisition.

2.8.3 Intracellular Staining

Only cells used in the whole blood assay required intracellular staining. Post-viability, cells were incubated with BD CytoFix/CytoPerm buffer at 4°C for 20 minutes. After incubation, cells were washed twice with 1x BD CytoPerm wash buffer using centrifugation. Post fixation, the cells were incubated with 50 μL of the intracellular staining panel listed in Tables 2.4 or 2.5 for 20 minutes at 4°C in the dark. Cells were washed twice with 1x BD CytoPerm wash buffer by centrifugation and resuspended in 3% paraformaldehyde. Cells were kept at 4°C in the dark until acquisition.

2.8.4 Flow Cytometry Staining Panels

Table 2.2 Fc Receptor Panel - Monocytes and DCs

Marker/Clone	Fluorophore	Volume per sample
Viability Dye	Aqua	N/A
CD3, UCHT1	V450	2.5 μL
CD14, TüK4	Qdot605	1 μL
CD16, 3G8	Pacific Orange	2.5 μL
CD32, FLI8.26	APC	10 μL
CD64, 10.1	APC H7	5 μL
CD89, A59	PE	5 μL
CD11c, B-ly6	A700	5 μL
CD123, 9F5	PECy5	5 μL
CD19, HIB19	FITC	5 μL
PBS	N/A	7.5 μL

Table 2.3 Fc Receptor Panel - NK Cells and Granulocytes

Marker/Clone	Fluorophore	Volume per sample
Viability Dye	Aqua	N/A
CD3, UCHT1	V450	2.5 μL
CD15, W6D3	BV650	2.5 μL
CD45, HI30	A700	2.5 μL
CD56, HCD56	PECy5	1.25 μL
CD16, 3G8	PECy7	2.5 μL
CD32, FLI8.26	APC	10 μL
CD64, 10.1	APC H7	5 μL
CD89, A59	PE	5 μL
CD66b, G10F5	FITC	2.5 μL
PBS	N/A	16.25 μL

Table 2.4 Whole Blood Assay Panel

Marker/Clone	Fluorophore	Volume per sample
Viability	Aqua	N/A
CD3, OKT3	PECy7	2.5 μL
CD4, RPA-T4	PE	2.5 μL
CD8, RPA-T8	APC	5 μL
p24, KC57	FITC	2.5 μL
BD CytoPerm Buffer	N/A	37.5 μL

Table 2.5 Antibody Binding Panel

Marker/Clone	Fluorophore	Volume per sample
Viability	Aqua	N/A
α-IgG, Polyclonal	A647	1 μg/mL
CD3, UCHT1	V450	2.5 μL
CD4, RPA-T4	PE	2.5 μL
p24, KC57	FITC	2.5 μL
BD CytoPerm Buffer	N/A	42.5 μL

2.8.5 Flow Cytometry Acquisition and Data Analysis

All samples were acquired on a BD FortessaLSR equipped with 50 mW 405 nm, 50 mW 488 nm, 50 mW 561 nm, 20 mW 633 nm lasers. Data was analysed using FlowJo (TreeStar, Ashland, OR, USA). Boolean gating plots for presentation purposes were made using data obtained in FlowJo and transferring it to SPICE 5.3, via PESTLE 1.7 (M. Roederer Vaccine Research Center at NIAID, MD, USA).

2.9 Cellular Inhibition Assays

2.9.1 Antibodies Screened

Table 2.6 Antibodies Screened and/or used in the Inhibition Assays and/or Whole Blood Assay.

Ab ID	Isotype	Epitope
17b	G1	CD4i (Cluster C)
2F5	**G1/dA2**	**gp41 (MPER)**

2G12	**G1**	**gp120 V3**
4B3	**G1**	**gp41 (Cluster I)**
4E10	**G1**	**gp41 (MPER)**
7B2	G1/mA2/dA2	gp41 (Cluster I)
7B2 AAA	G1	gp41 (Cluster I)
A32CL4	G1	CD4i C1 (Cluster A)
b12	**G1**	**CD4bs**
CH01	G1	gp120, V1V2/V2V3
CH20	G1	CD4i C1; non-A32 blockable
CH22 AAA	G1	gp120 V3, linear
CH23	G1	gp120 V3, linear
CH27 AAA	G1/A2	Consensus C.Con gp140 Env
CH28	G1/A	DU123/Con-S A2, VH3-66
CH29	G1/A2	CD4i C1, A32-blockable
CH31	**G1/mA2/dA2**	**CD4bs, VRC01-like, bnAb**
CH38	G1/A1	CD4i C1, A32-blockable
CH54	G1	CD4i C1, A32-blockable
CH55	G1	CD4i C1, A32-blockable
CH57	G1	CD4i C1, A32-blockable
CH58	G1	gp120, V2
CH59	G1	gp120, V2
CH65	G1/A2	HA, H1 - Flu Control Ab
CH90 AAA	G1	CD4i C1, A32-blockable
CH115	G1/A1	gp41
F240	G1	gp41 (Cluster I)
HG107	G1	gp120, V2
HG116	G1	gp120
HG118	G1	gp120
HG120	G1	gp120, V2
HG129	G1/A1	C1 linear peptide
HG130	G1/A1	Consensus S gp140 Env.
HIVIG B	G	Clade B polyclonal IgG
HIVIG C	G	Clade C polyclonal IgG
M785-U1	G1	gp41
N10-U1	G1	gp41
PG9	**G1**	**gp120, V1V2; Quaternary Structure**
PG16	**G1**	**gp120, V1V2; Quaternary Structure**
VRC01	**G1**	**CD4bs**

Neutralising antibodies in bold; Antibodies used in the Inhibition Assays and Whole Blood Assay in grey; CD4i = CD4 inducible; AAA = Fc region modified to increase Fc receptor binding.

2.9.2 MDM Inhibition Assay

Antibodies were prepared at 100 μg/mL in AIM-V media and incubated 1:1 with cell free HIV-1_{BaL} (10^4 $TCID_{50}$) for 1 hour at 37°C. The virus-antibody suspension was added onto 5 day-old macrophage cultures and incubated for 2 hours at 37°C. Post-incubation, cells were washed 3 times with PBS and antibodies at 50 μg/mL in 200 μl AIM-V media + 20 ng/mL GM-CSF. The cells were further incubated for 7 days in a 95% humidified incubator with 5% CO_2 at 37°C.

All assays were performed in triplicate and included controls: medium only, virus only and antibody isotype controls at the same concentration as the test antibodies (Figure 2.1a).

2.9.3 MDDC Inhibition Assay

Antibodies were prepared at 100 μg/mL in complete RPMI media and incubated 1:1 with cell free HIV-1_{BaL} (10^4 $TCID_{50}$) for 1 hour at 37°C. 4 x 10^4 MDDC were added in 100 μL complete RPMI medium to the virus antibody suspension and incubated overnight 37°C. Post-incubation, cells were washed 3 times with PBS by centrifugation and antibodies were added at 50 μg/mL in 200 μL complete RPMI media + 30 ng/mL IL-4 and 25 ng/mL GM-CSF. The cells were further incubated for 7 days in a 95% humidified incubator with 5% CO_2 at 37°C.

All assays were performed in triplicate and included controls: medium only, virus only and antibody isotype controls at the same concentration as the test antibodies (Figure 2.1a).

2.9.4 MDDC-PM-1-T cells Trans-infection Inhibition Assay

MDDC at 2x 10^4 cells per well were incubated with cell free HIV-1_{BaL} (10^4 $TCID_{50}$) for 1 hour at 37°C. Post-incubation, MDDC were washed 3 times with PBS by centrifugation and 50 µg/mL antibody was incubated with the cells for 30 minutes at 37°C. Post-incubation, 4 x 10^4 PM-1 T cells were added per well and the cells were further incubated for 7 days in a 95% humidified incubator with 5% CO_2 at 37°C.

All assays were performed in triplicate and included controls: medium only, virus only and antibody isotype controls at the same concentration as the test antibodies (Figure 2.1b).

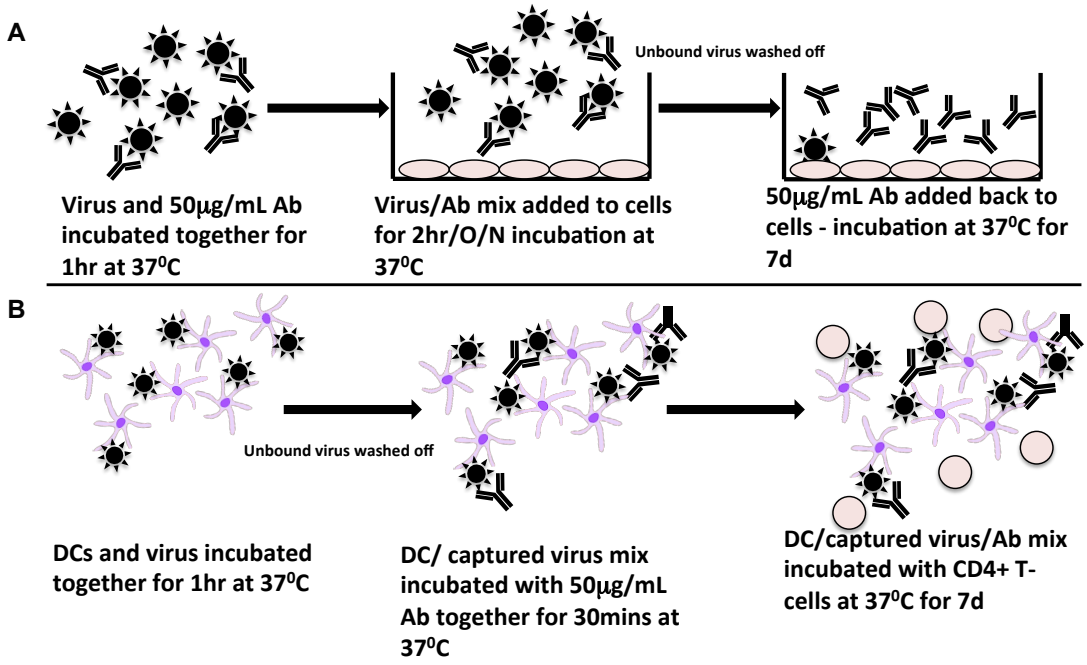


Figure 2.1 Inhibition assay setup in MDM and MDDC (A) or MDDC-T cells (B)

2.10 Human Mucosal Tissue Explant Inhibition Assays

2.10.1 Mucosal Tissue Explant

Antibodies were prepared at 100 μg/mL in complete RPMI media and incubated 1:1 with cell free HIV-1_{BaL} ($2x10^4$ $TCID_{50}$) for 1 hour at 37°C. Tissue explants were cut to 3 mm^3 and added to the virus/antibody suspension for overnight incubation at 37°C. Post-incubation, tissues were washed 3 times with PBS and antibodies were added at 50 μg/mL in 200 μL complete RPMI media. The tissues were further incubated in a 95% humidified incubator with 5% CO_2 at 37°C for a total of 21 days. Every 3-4 days 100 μL of supernatant was removed and replaced with 100 μL fresh complete RPMI.

All assays were performed in triplicate and included controls: medium only and antibody isotype controls at the same concentration as the test antibodies (Figure 2.2a).

2.10.2 Mucosal Migratory Cells

Post-overnight infection, migratory cells were collected from the tissue explants and washed three times with PBS by centrifugation [381, 382]. The cells were further incubated with 50 μg/mL antibody and 4 x 10^4 PM-1 T cells for 7 days in a 95% humidified incubator with 5% CO_2 at 37°C (Figure 2.2b).

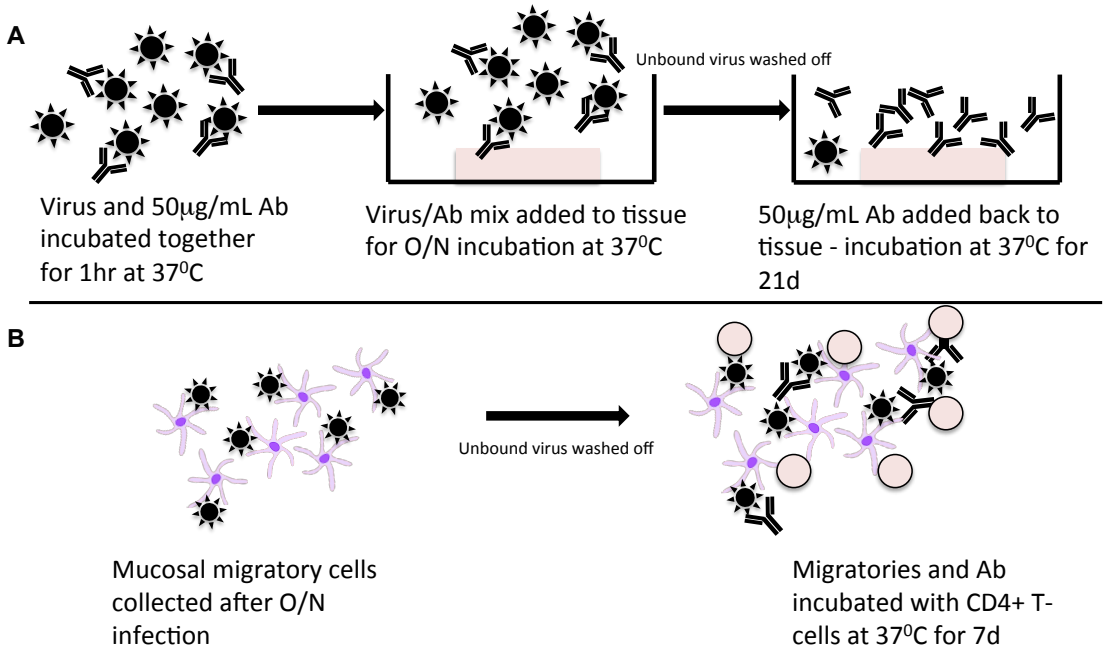


Figure 2.2 Inhibition assay setup in mucosal tissue explants (A) and migratory cell-T cells (B)

2.11 TZM-bl Neutralisation Assay

TZM-bl cells were utilised to assess the potential neutralising ability of antibodies against HIV-1_{BaL} [380]. Test antibodies were titrated 1:4 from a starting concentration of 40 μg/mL in final volume of 50 μL in triplicate wells of a 96-well flat bottom culture plate. TZM-bl cells were prepared at 10^5 cells/mL and 100 μL cells were added the antibody titrations. Finally, 50 μL of HIV-1_{BaL} was added at a concentration previously determined by titration on TZM-bl cells. Positive control (virus + cells only) and negative control (cells only) wells were included in the assay setup. The cells were incubated in a 95% humidified incubator with 5% CO_2 at 37°C for 36-72 h.

Post-incubation, all supernatant was removed from the cells, which were washed once with 200 μL PBS. Luciferase lysis buffer was diluted 1:5 with dH_2O and 100 μL was added to all wells. Plates were placed at -80°C for at least 2 h to allow for full lysis.

Post-lysis, the plates were thawed and 50 μL of the lysate was transferred to a white 96-well high-bind plate. Luciferase substrate was reconstituted by adding 10 mL luciferase buffer to the lyophilised substrate. 50 μL of luciferase substrate was added to the lysate and mixed well. Plates were read immediately in relative light units (RLU) using a FLUOstar Omega plate reader (BMG Labtech, UK).

2.12 AALTO HIV-1 p24 Antigen ELISA

AALTO HIV-1 p24 antigen ELISA was used as an end-point read out to test supernatants derived from the cellular and mucosal explant inhibition assays. White high-bind 96-well plates were coated with 100 μL 5 μg/ml p24 coating antibody and incubated overnight at RT. Post-incubation, plates were washed twice with 1x TBS and blocked using 100 μL/well 2% whole milk for 1 h at RT. Post-block, plates were frozen at -20°C until required.

Coated p24 plates were thawed and washed once with 1x TBS and lysed HIV-1 (MN) CL-4 H9 was used as a standard, starting concentration 10 ng/mL, 1:2 serial dilution in 0.1% Empigen TBS.

HIV-1 positive samples were first treated with 1% Empigen to inactivate the virus and 100 μL final volume of sample was added to the coated ELISA plate for 2-3 h incubation at RT.

Plates were washed twice with 1x TBS and 100 μL 1:8000 conjugate was added per well for 1 h incubation at RT. Plates were washed four times with 0.05% PBST and then two times with ELISA Light assay buffer. CSPD with Sapphire Substrate was diluted 1:3 with ELISA light assay buffer and 50 μL/well

of the substrate was added. Plates were incubated for 20-30 minutes at room temperature and relative light units (RLU) were read using FLUOstar Omega plate reader (BMG Labtech).

2.13 RETROTek HIV-1 p24 Antigen ELISA

For more sensitive detection of HIV-1 p24, the RETROTek ELISA kit was utilised as per manufacturers' instructions, as the detection range for this assay is between 4 – 125 pg/mL.

2.14 Whole Blood Assay

2.14.1 PBMC Stimulation and CD4+ Cell Isolation

Fresh PBMC were isolated from HIV-negative donors, as described in 2.4.1. The PBMC were stimulated for 72 h in 40 mL complete RPMI supplemented with 5 μg/mL PHA-L and 10 U/mL IL-2. Post-stimulation, the CD4+ T cells were isolated from the PBMC using the CD4+ dynabead isolation kit. PBMC were resuspended at $1x10^7$ cells/mL in 2% FBS/PBS. CD4+ dynabeads were washed once in 2% FBS/PBS and used at 25 μL/mL cells. PBMC and CD4+ dynabeads were incubated together at 4°C with gentle tilting and rotation for 90 minutes.

Post-incubation, the sample was placed in a DynaMag -15 magnet for 2 minutes and, with the tube still in the magnet, the supernatant was removed and discarded. The tube was removed from the magnet and 1 mL 2% FBS/PBS was added to wash the cells. The tube was placed back into the magnet for a further 2 minutes and the supernatant discarded. This process was repeated once more to

improve the purity of the CD4+ population and the cell pellet was resuspended in 1 mL complete RPMI.

To remove the dynabeads from the CD4+ cells, the samples were further incubated with 10 μL DetachaBead per $1x10^7$ cells (starting volume) at RT for 45 minutes. Post-incubation, the cells were placed on the magnet for 1 minute and the supernatant was collected and placed in a fresh tube. Residual cells were collected through washing the beads 2-3 more times with 1 mL complete RPMI, collecting the supernatant each time. The CD4+ T cells were washed once in 15 mL complete RPMI, counted and resuspended at the desired concentration for use in the whole blood assay.

2.14.2 Staining with Cell Trace Violet (CTV)

CD4+ T cells were resuspended at a concentration of $1x10^6$/mL in PBS 5% FBS in a 50 mL falcon tube. CTV was reconstituted in DMSO to a final concentration of 5 μM and 1 μL was added for every $1x10^6$ CD4+ T cells. The CTV was added to the side of a falcon tube tilted to a 45° angle. The falcon tube was quickly inverted and vortexed to ensure consistent staining. The cells were incubated in the dark for 20 minutes with constant rotation. Post incubation, the falcon tube was topped up to 50 mL with complete RPMI and incubated in the dark with rotation for a further 5 minutes. Cells were washed once with complete RPMI and resuspended in complete RPMI supplemented with 10 U/mL IL-2.

2.14.3 HIV_{BaL} Infection

CD4+ T cells were resuspended in complete RPMI supplemented with 10 U/mL IL-2 at a concentration of $1x10^6$ – $3x10^6$ cells/mL. 100 μL of cells were

added per well and 100 μL of HIV_{BaL} (grown in PBMC) was added to the cells at a 1:5 final dilution ($2x10^3$ $TCID_{50}$). Cells and virus were co-incubated for 7 days in a 95% humidified incubator with 5% CO_2 at 37°C.

2.14.4 Whole Blood Assay Setup

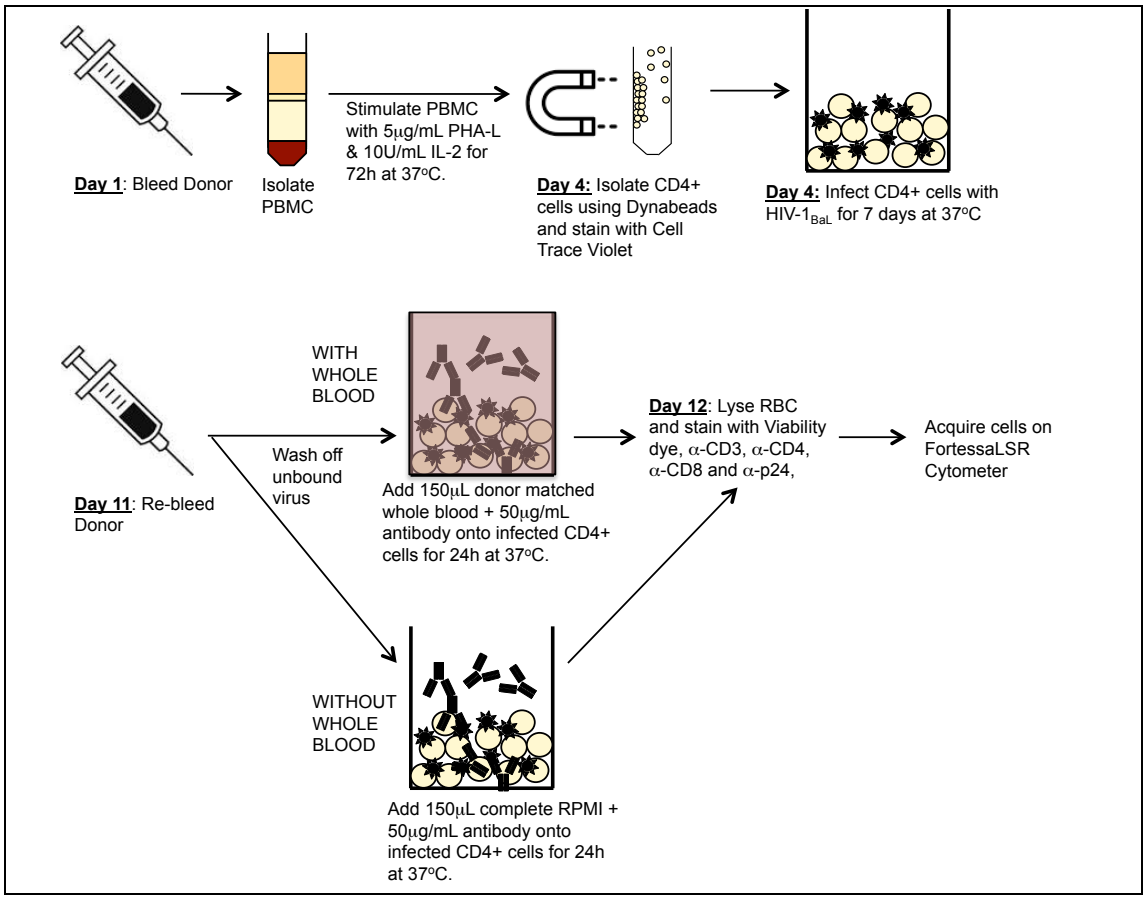


Figure 2.3 Schematic of the Whole Blood Assay

CTV+ CD4+ T cells were infected with $HIV\text{-}1_{BaL}$, as described in 2.14.3. Post-infection, the cells were co-incubated with the panel of antibodies, outlined in Table 4.3, at 50 μg/mL final volume and 150 μL donor-matched whole blood (WB) for 24 hours in a 95% humidified incubator with 5% CO_2 at 37°C. Post-incubation, the red-blood cells (RBC) were lysed using 200 μL ACK lysis buffer for 5 minutes at room temperature. Cells were washed twice with PBS and stained with Aqua, as described in 2.8.1. Post-incubation, cells were stained with

flow cytometry panel listed in Table 2.4, as described in section 2.8.3, resuspended in 3% formaldehyde and kept at 4°C in the dark until acquisition (Figure 2.3).

2.14.5 HIV-1 Antibody Binding Experiments

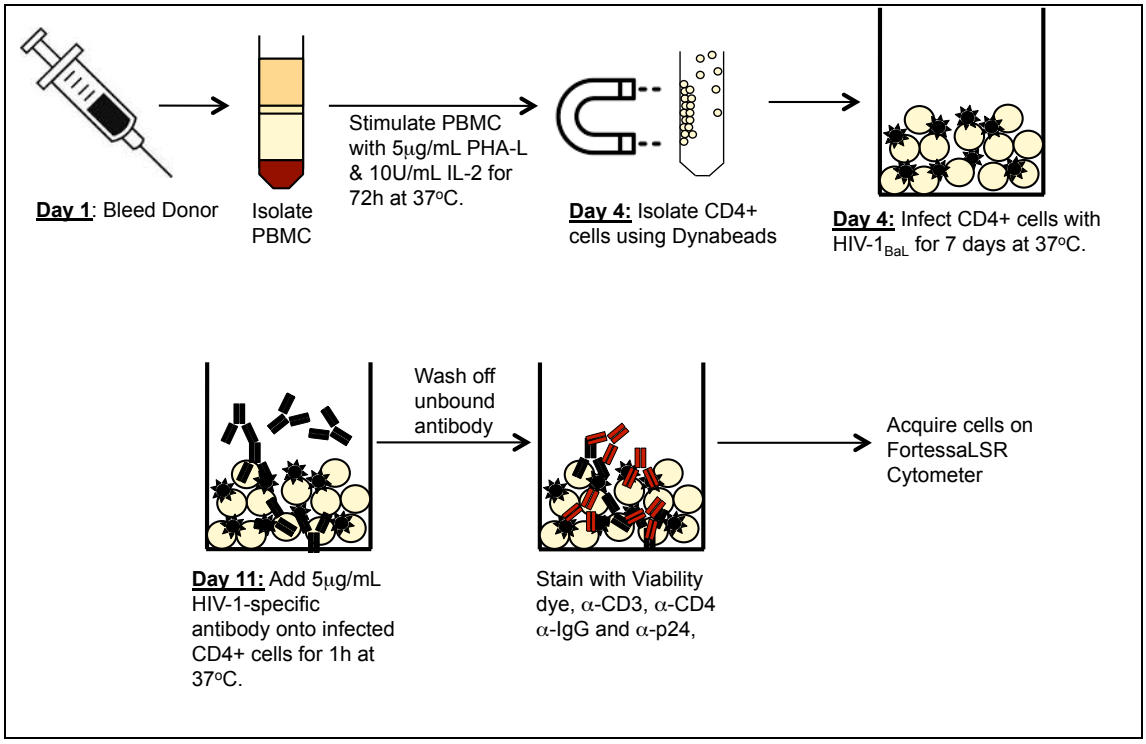


Figure 2.4 Schematic for the detection of antibody binding

CD4+ T cells were infected with HIV-1_{BaL}, as described in 2.14.3. Infected cells were then stained with Aqua viability dye, as described in 2.8.1, before being incubated with 5 μg/mL HIV-1 antibody chosen from the panel outlined in Table 4.3 for 1 hour in a 95% humidified incubator with 5% CO_2 at 37°C. Post-incubation, cells were stained for 20 minutes at room temperature with the flow cytometry panel listed in Table 2.5, as described in section 2.8.3, resuspended in 3% formaldehyde and kept at 4°C in the dark until acquisition.

2.15 Statistical Analysis

All statistical analyses were performed using Prism 7.0 (Graphpad, USA). For non-parametric data, statistical analyses were performed using Kruskal-Wallis test with Dunn's multiple comparison correction. For parametric data, statistical analyses were performed using one-way ANOVA with Dunnett's multiple comparison correction. $P<0.05$ was considered significant: *$P<0.05$; **$P<0.01$; ***$P<0.001$; ****$P<0.0001$.

CHAPTER 3 – RESULTS:

Characterisation of Fc Receptor Expression within Mucosal Tissues Associated with HIV-1 Transmission

3 Characterisation of Fc Receptor Expression within Mucosal Tissues Associated with HIV-1 Transmission

3.1 Introduction

In recent years, interest in nnAb to HIV-1 and their associated effector functions has increased. This interest is largely due to post-trial analysis performed on RV144 vaccine participant samples, which demonstrated that the partial protective efficacy conferred by this vaccine could be correlated with high levels of ADCC-inducing IgG3 antibodies to the V1V2 region of Env [134, 353, 355, 358, 375, 383, 384]. This positive IgG response was negated in the presence of IgA antibodies to the same region of Env. Furthermore, studies have revealed that nAb activity is enhanced by FcR effector functions, broadening the importance of these receptors in the prevention of HIV-1 infection [385-387].

For ADCC and other antibody-effector functions to occur, the Fc portion of an antigen-bound antibody must bind to its associated FcR, found on the surface of immune effector cells. Although some FcR are expressed by B cells [388], they are predominantly found on the surface of innate effector cells, such as NK cells, macrophages, DCs and neutrophils [146]. As such, FcR are an integral component in bridging the gap between innate and adaptive immune responses.

Once bound to an antigen, antibodies complex together leading to localised clustering of FcR on the cell membrane. It is this clustering that initiates antibody-dependent cellular effector functions. In terms of HIV-1, antibody complexes are formed when infected cells and/or cells coated with viral particles are opsonised by HIV-1 specific antibodies [389]. The protective abilities conferred by antibody-dependent cellular effector mechanisms are dependent

on a variety of parameters. The effector cell type and frequency, the allotype of FcR expressed, the frequency of FcR per cell, and the glycosylation pattern of the induced antibodies are all capable of impacting the functional activities of FcR. Furthermore, FcR can be either inhibiting or activating, which, depending on the FcR in question, can be attributed to their association with immunoreceptor tyrosine-based inhibitory (ITIM) or immunoreceptor tyrosine-based activation (ITAM) motifs. For example, despite being almost identical in structure, CD32A and CD32B differ in their functional activities due to their relative associations with the ITAM and ITIM motifs, respectively [146, 390].

ADCC is activated most efficiently through CD16A, predominantly expressed on NK cells, but also found on other immune cells such as neutrophils, and subpopulations of monocytes, macrophages and DCs [132, 140]. Upon interaction of the opsonised cell and CD16A-expressing NK cells, the contents of cytotoxic granules including perforin, granzymes and cytokines such as IFN-γ, are released in a directional manner across a lytic synapse leading to cytolysis of the opsonised cells.

ADCC is not the only antibody-dependent effector function that may have a protective role against HIV-1 transmission. ADCP and ADCVI have both demonstrated activity against HIV-1 [391]. The primary mode of activity for ADCP is through interaction between the Fc region of an HIV-1 specific antibody and FcR CD32A, CD64 and CD89. These FcR are found on the surface of professional phagocytic cells such as monocytes, macrophages and DCs. When the FcR binds the opsonised virally infected cells, they are engulfed by the phagocytic cells and subsequently degraded [173] [151]. ADCVI is specifically associated with virally infected cells and is a combination of ADCC and ADCP

activity with the additional release of HIV inhibitory β-chemokines, which block onward infection of susceptible cells [176-178].

ADCC can also be triggered through engagement of CD64 and CD32 on myeloid cells (monocytes, macrophages and DCs) [140, 163]. However, the efficiency of ADCC induced by myeloid cells contrasts when compared to ADCP, which for myeloid cells is reported to occur rapidly within 1-4 h, versus up to 24 h for ADCC [175].

Sexual transmission of HIV-1 via mucosal surfaces, particularly those of the rectal mucosa and male and female genital tracts account for around 90% of newly acquired infections [289]. Despite this, comparatively little is known about the composition of immune cells found within these tissues or their potential to interact with antibodies in defending against pathogenic infections. Therefore, critical to vaccine design is the characterisation of the phenotype and frequency of antibody effector cells present within mucosal surfaces most commonly associated with HIV-1 transmission.

Past research from the early 1990s used RNA and histological techniques to determine that CD16 and CD32 were expressed on colorectal and cervical mucosa [142-145]. Further characterisation of cervical tissue has been performed in more recent years using flow cytometry methods, although these investigations did not look at FcR expression, but solely focused on immune cell phenotype [145, 265]. The most comprehensive study to date, which combined flow cytometry techniques to investigate immune effector cell phenotype and FcR expression with additional immunohistology to determine the location of effector cells within tissue, did so using colorectal and cervical tissue only [174]. Significantly, all studies performed so far neglect to define the effector cells and

FcR expression found within penile tissue, which is an important site of HIV-1 transmission.

The aim of this chapter is to determine the immune effector cell and FcR expression profile within mucosal tissues associated with HIV-1 transmission, specifically penile glans, ectocervical and colorectal tissue. In this chapter, results from flow cytometric analysis of cells isolated from the three different mucosal tissue types will be compared to determine immune effector cell phenotype and frequency. In addition, expression of IgG (CD16, CD32 and CD64) and IgA (CD89) FcR on CD14+ monocytes, myeloid DCs and NK cells will be assessed. All data from mucosal tissues will be compared to data from cells isolated from red-blood cell-lysed whole blood. Furthermore, deconvolution immunofluorescent microscopy will be used to identify the location of immune effector cells and their associated FcR within the three types of mucosal tissue.

The data obtained here will be valuable to inform on whether immune cells found within mucosal tissues are capable of inducing an immune response in the presence of nnAb to HIV-1 that are functionally active through the Fc-portion.

This chapter has formed the basis of a paper, which has been published in PLoS One [392].

3.2 Results

3.2.1 Tissue Digestion and Experimental Setup

Before any characterisation could take place using flow cytometry, it was important to develop a protocol that would efficiently isolate cells from mucosal tissue without degrading cell surface markers.

3.2.1.1 Titration of Liberase DL on PBMC

A titration of Liberase Dispase Low (DL) was performed on PBMC to determine the concentration at which surface expression of vulnerable markers, such as CD4 and CD8, was lost. The data showed that detection of the CD4+ receptor began to reduce at 25 μg/mL, therefore a concentration of 12.5 μg/mL was used for the next stage (Figure 3.1A).

3.2.1.2 The Effect of Hyaluronidase and DNase on PBMC

Based on a literature search, additional digestion reagents, hyaluronidase and DNase, were added to the final enzyme cocktails at 25 μg/mL and 100 μg/mL, respectively, to maximise cellular recovery [393, 394]. An assessment of the effect of these digestion reagents on CD4+ and CD8+ markers was made using PBMC. The results demonstrated that there was no adverse reduction in expression of either of these markers (Figure 3.1B), so they were included in the final digestion cocktail.

3.2.1.3 Liberase Selection on Penile Glans and Colorectal Tissue

A panel of Liberase enzymes (Dispase Low (DL) or High (DL) and Thermolysin Low (TL), Medium (TM) or High (TH)) were tested on colorectal and penile glans tissue to determine the best recovery of markers CD4 and CD8. The results indicated that Liberase DL at 12.5 μg/mL was the best enzyme to use

in colorectal tissue (Figure 3.1C) as it yielded the best recovery of CD4+ and CD8+ cells. For penile tissue, a titration of the Liberase enzymes that had shown loss of the CD4+ and CD8+ markers at 12.5 μg/mL was performed to assess if they would yield better recoveries at lower concentrations (Figure 3.1D). However, these enzymes continued to exhibit CD4+ receptor loss at low concentrations and their use was discontinued at this point. The best recovery of CD4+ and CD8+ cells was noted when using Liberase TL at 12.5 μg/mL (Figure 3.1D), therefore this was the enzyme and concentration selected for future penile tissue digestion. Due to low availability of tissue, no comparison was made for ectocervical tissue, however, as this tissue is more structurally similar to penile tissue than colorectal tissue [395], it was decided that Liberase TL should also be used for digestion of this tissue type.

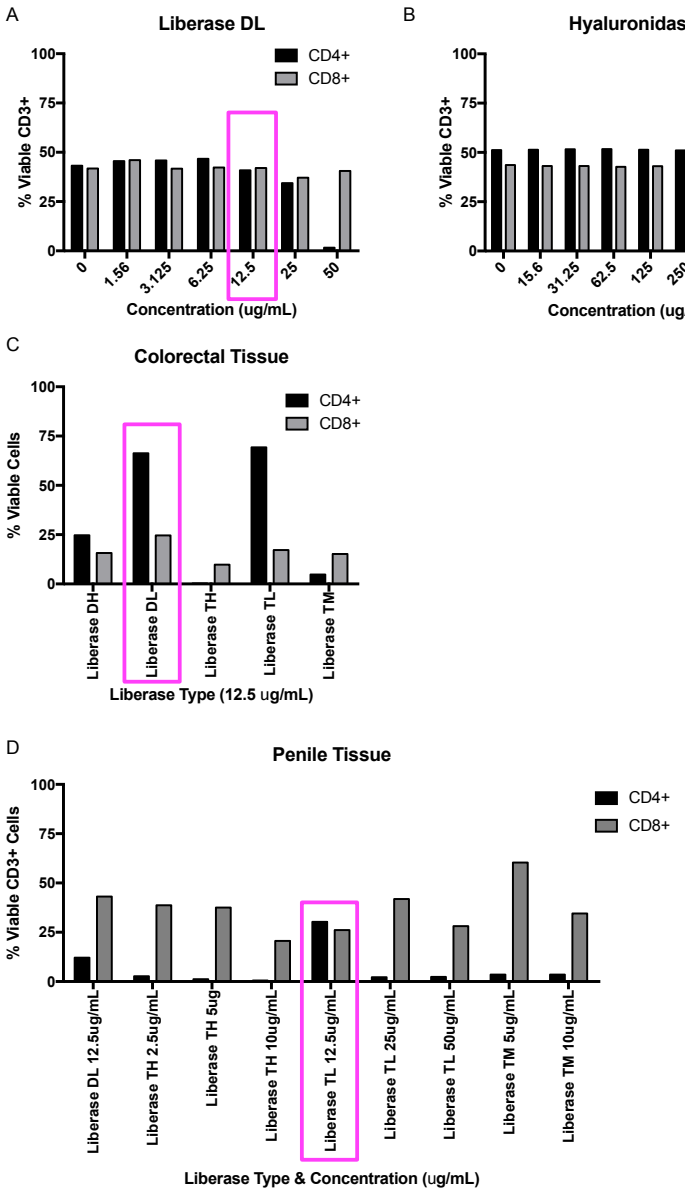


Figure 3.1 Liberase Selection and Titration in PBMC, Colorectal and Penile Tissue.

(A) Liberase DL titrated on PBMC to determine the concentration at which vulnerable markers were degraded. (B) Hyaluronidase titration on PBMC in the presence of 12.5 μg/mL Liberase DL and 100 μg/mL DNase. (C) A panel of Liberase enzymes [Liberase Dispase Low (DL), Dispase High (DH), Thermolysin Low (TL), Thermolysin Medium (TM) and Thermolysin High (TH) were screened on colorectal tissue at a concentration of 12.5 μg/mL and relative expression of CD4+ and CD8+ markers were assessed. (D) The same panel of Liberase enzymes were used to digest penile glans tissue at a range of concentrations. (Pink = Concentrations/Enzyme selected)

3.2.2 Flow Cytometry Panel Development

To enable the characterisation of FcR expression, two flow cytometry panels were designed. The design of these panels allowed for characterisation of immune effector cells isolated from mucosal tissues before expanding to identify the expression of CD16, CD32, CD64 and CD89 on these effector cells.

3.2.2.1 FACS Antibody Selection and Titration

The FACS antibody selection was based on knowledge of surface marker expression and fluorochrome brightness, where the brightest fluorochromes were used for low-level marker expression and vice versa. Details of the staining panels and procedures are outlined in section 2.8.

All FACS antibodies were titrated on PBMC to obtain the optimal staining concentration. Briefly, a starting titration of double the recommended concentration was added to the cells in a final staining volume of 50 μL. A 2-fold serial dilution was then performed. To ensure there was no bleed-over from other staining channels during acquisition of the PBMC, only the channel of interest was opened for each fluorochrome. The chosen concentrations were those with the greatest difference in MFI between the positive and negative populations (Figure 3.2).

3.2.2.2 Fluorescence Minus One (FMO) Experiments

Fluorescence minus one (FMO) experiments, designed to allowed correct interpretation of the flow cytometry data, were also performed in PBMC. Briefly, the cells were stained with the full antibody panel with one fluorochrome sequentially removed to enable accurate determination of where gates should be

placed (Figure 3.3). The data generated here allowed for the gating strategies set out in Figure 3.4 to be used to analyse all subsequent experiments.

3.2.2.3 Effects of Liberase Digestion on Markers of Interest

To ensure there were no adverse effects of the digestion process on any markers of interest, full FACS panels were used to stain RBC-lysed whole blood in the presence or absence of the complete enzyme digestion cocktail (Figure 3.5). Results demonstrated that expression levels of all markers of interest remained comparable across all conditions, therefore confidence could be taken that the digestion process did not influence expression profiles of digested tissues.

3.2.2.4 Ability of FcR FACS Antibodies to Bind in the Presence of Human Serum

Lastly, to have confidence that the FcR FACS antibodies were capable of binding their respective markers in the presence of human immunoglobulin, PBMC were stained with 0%, 10% or 20% human serum. Results showed no difference in the expression levels of CD16, CD32, CD64 or CD89 across the three concentrations demonstrating that any observed difference in FcR expression levels across the three tissue types were not simply artefacts of varying immunoglobulin levels found within these diverse mucosal compartments (Figure 3.6).

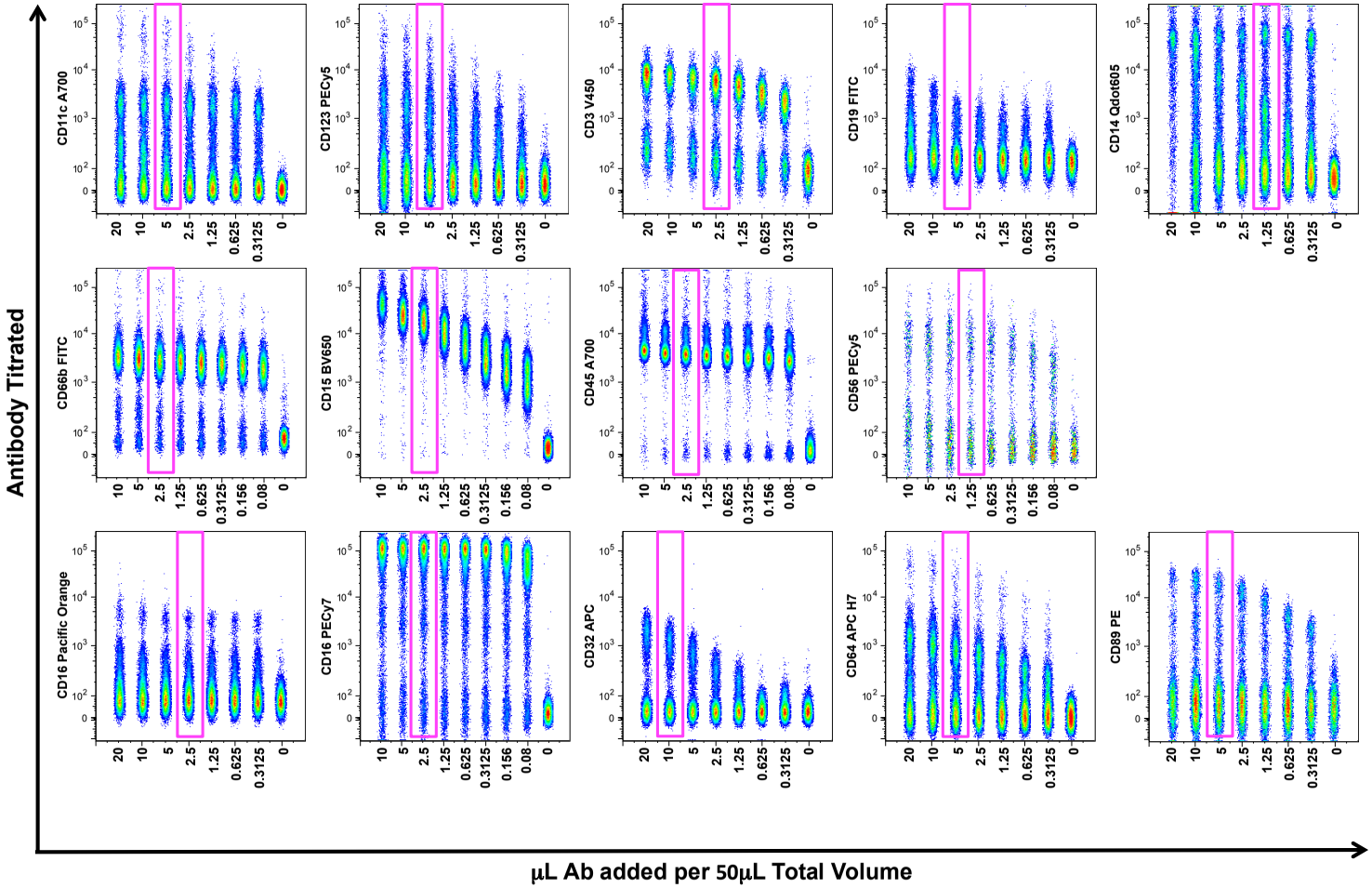


Figure 3.2 Concatenation of titrated FACS antibodies used in the Fc Receptor panels.

All antibodies were titrated in a total staining volume of 50 µL. Titrations used were from a starting amount that was double the manufacturers recommended concentration and a 2-fold dilution prepared. (Pink = Concentration selected)

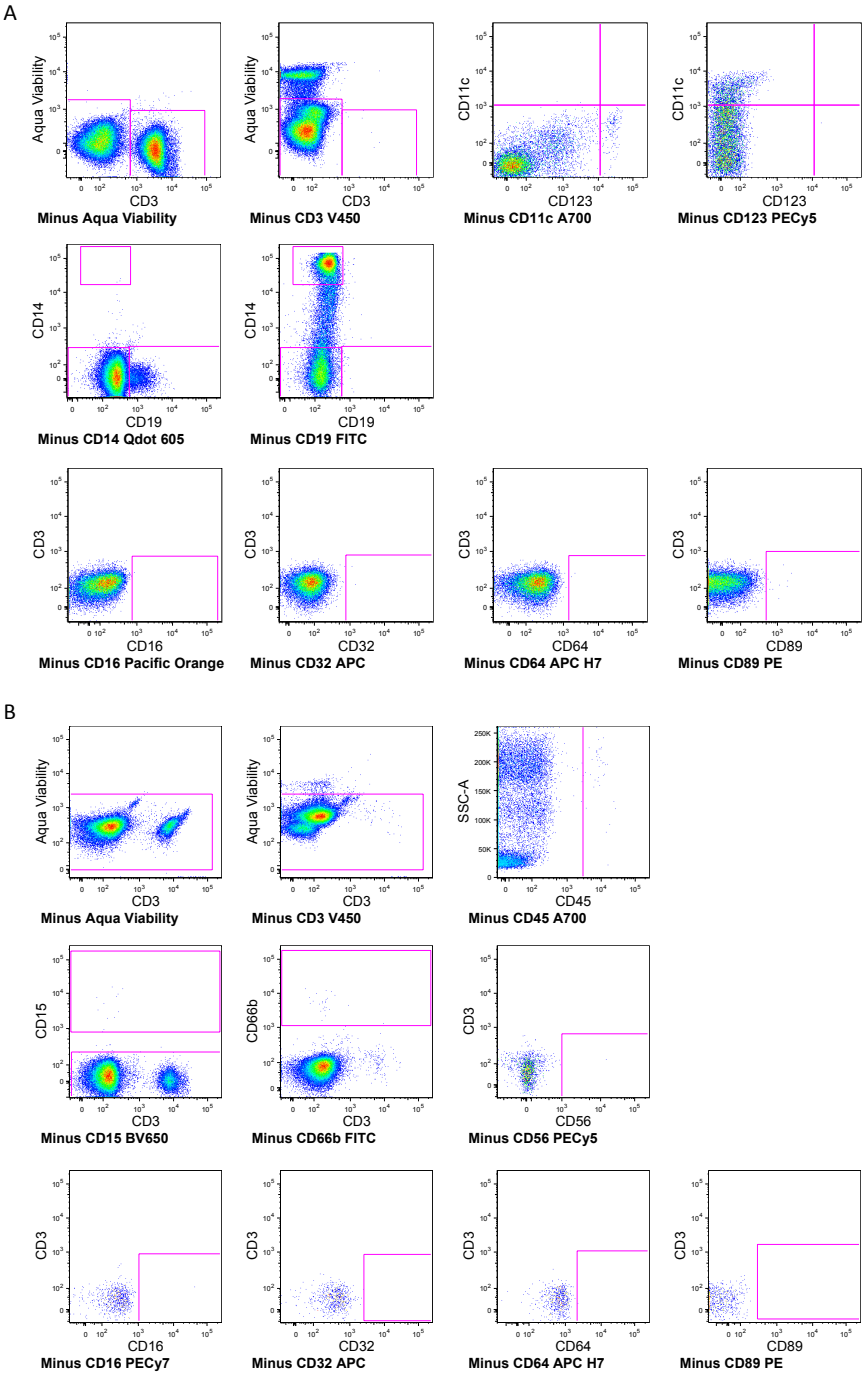


Figure 3.3 Fluorescence Minus One (FMO) Data.

FMO data was generated in PBMC to enable accurate placement of analysis gates in (A) FcR expression in CD14+ monocytic cells and mDC and (B) FcR expression in NK cells and granulocytes.

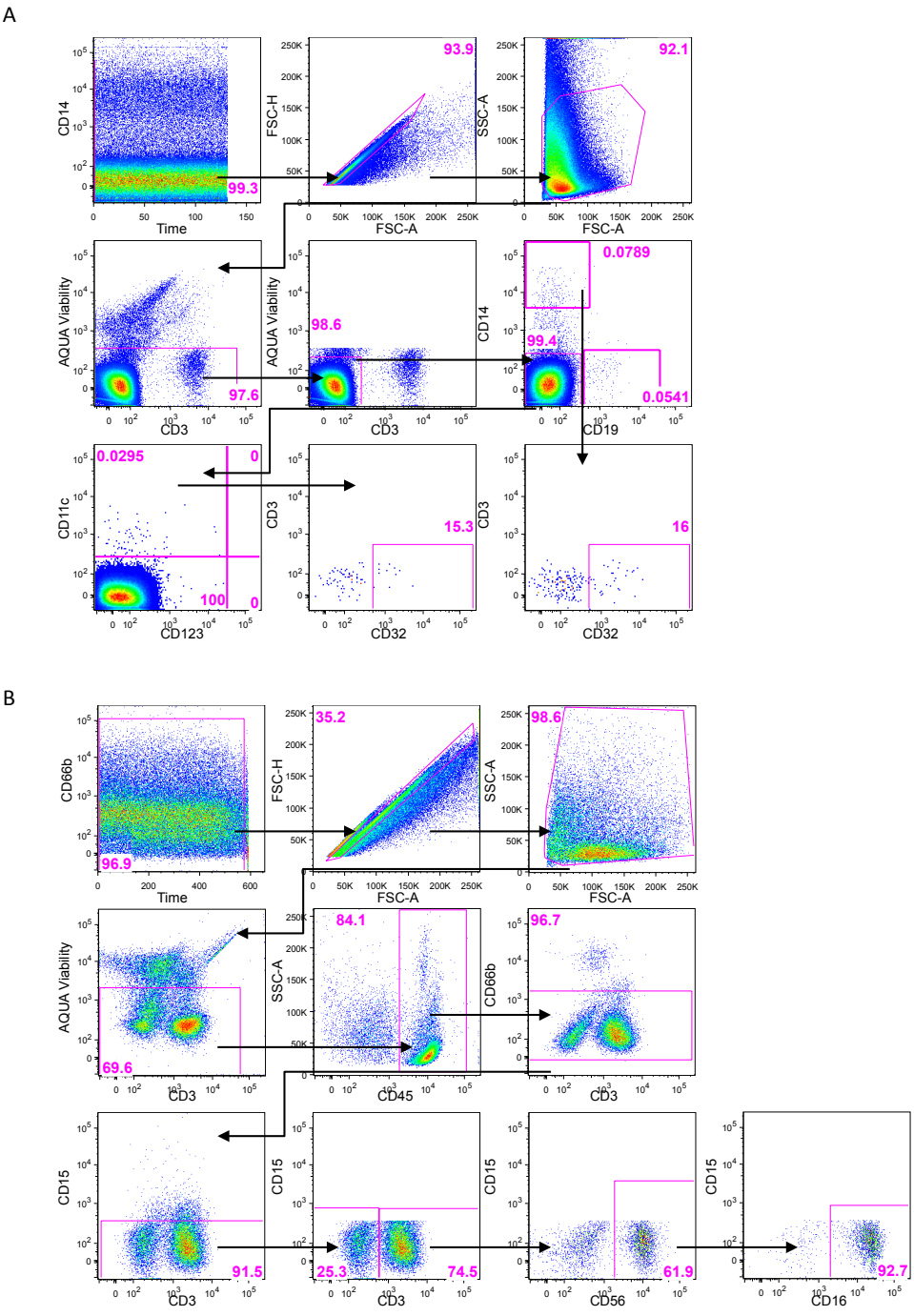


Figure 3.4 Flow Cytometry Gating Strategies used in FlowJo.

To analyse the data, a time gate was first applied to exclude any electronic noise. Following this, a singlet gate excluded any doublets and a viability gate excluded any dead cells. (A) FcR expression on monocytic CD14+ cells and mDC; CD14+ monocytic cells were defined as CD3-CD14+; mDC were defined as CD3-CD14-CD19-CD11c+. (B) FcR expression on NK cells and neutrophils. NK cells were defined as CD45+CD3-CD56+, whereas neutrophils were defined as CD45+CD3-CD15+CD66b+. All cell types were assessed for expression of CD16, CD32, CD64 and CD89 (data shows representative plots for cells isolated from penile glans tissue.)

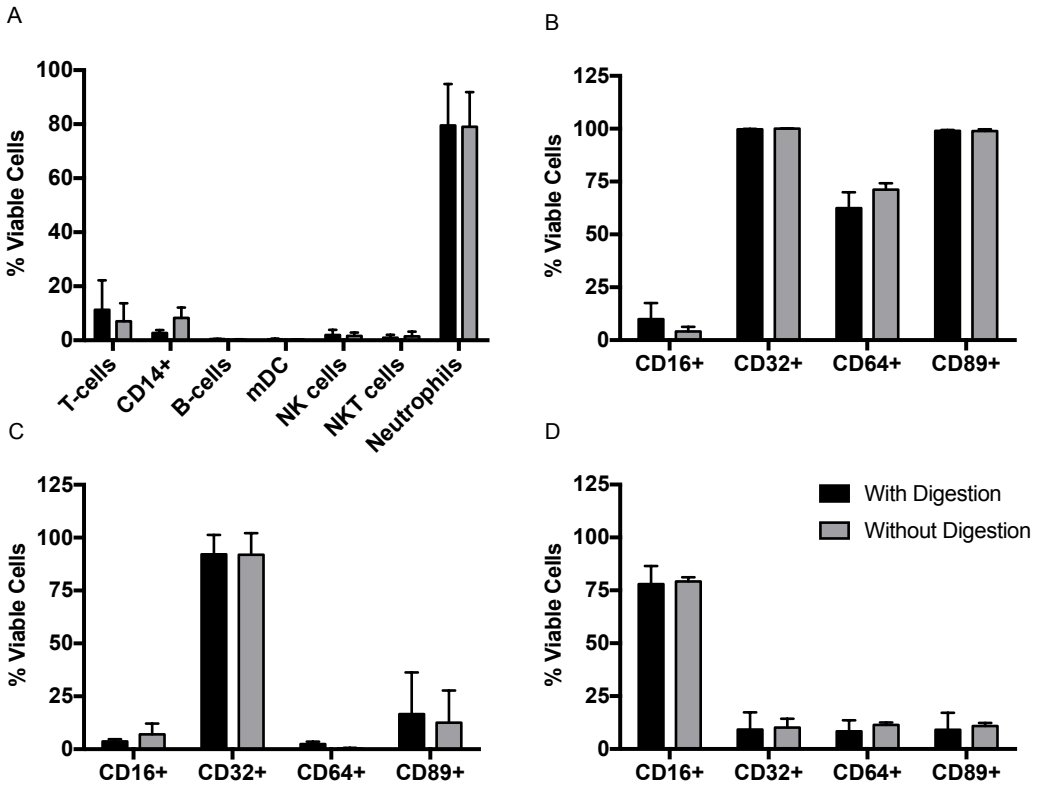


Figure 3.5 Flow cytometry analysis of RBC-lysed whole blood pre- & post-digestion with Liberase DL.

Cellular profiles and FcR expression for (A) Overall cellular profiles; (B) FcR expression on CD14+ monocytic cells; (C) FcR expression on mDC and (D) FcR expression on NK cells isolated from RBC-lysed whole blood (n=3).

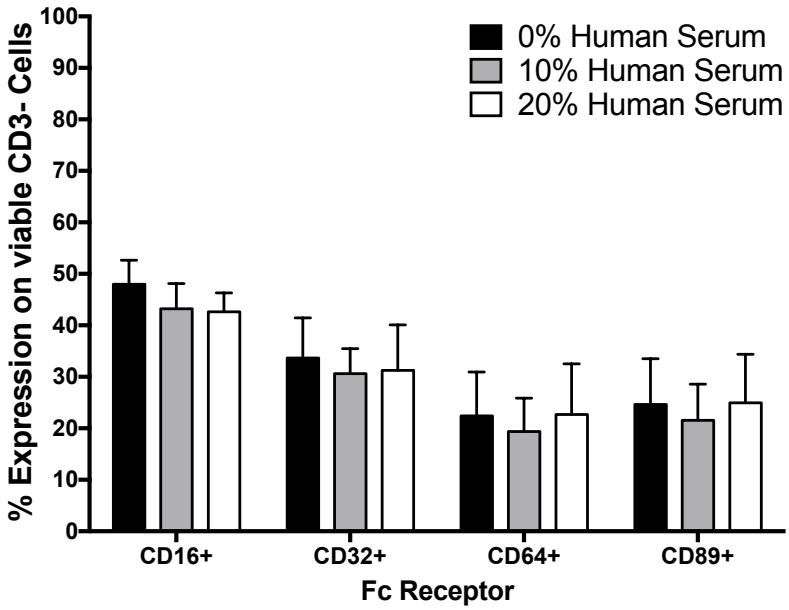


Figure 3.6 Effect of human serum concentration on FcR profiles in PBMC.

To assess the effect of human serum levels on the ability of FcR antibodies CD16, CD32, CD64 and CD89 to bind to their respective receptors, FACS staining cocktails with increasing levels of human serum were used to stain PBMC (n=3).

3.2.3 Characterisation of Immune Cell Phenotypes within Mucosal Tissues

Basic characterisation of immune cells isolated from penile glans, ectocervical and colorectal tissue was performed and compared to the immune profile found in cells isolated from red-blood cell-lysed whole blood. The use of RBC-lysed whole blood instead of PBMC was to ensure a direct comparison could be made between peripherally- and mucosally-isolated cells and to ensure neutrophils were included in the overall comparison.

Comparison of CD3+ T cells, CD19+ B cells, myeloid DCs (mDC), NK cells, NKT cells, CD14+ monocytic cells and neutrophils was made, with results indicating no significant difference between percentages of NKT cells, mDC or CD3+ T cells isolated from the three mucosal tissues and the whole blood. A trend towards higher expression of CD3+ T cells was observed for cells isolated from penile tissue, however, this did not reach statistical significance (Figure 3.7A).

Cells isolated from whole blood showed greater percentage expression of CD14+ monocytic cells when compared to cells isolated from penile glans ($P=0.046$) and ectocervical tissue ($P=0.037$) (Figure 3.7A). Although there was no statistical significance, a trend for an increased percentage of CD14+ monocytic cells isolated from colorectal tissue was noted when compared to the other mucosal tissue types (Figure 3.7B). CD19+ B cells were isolated from colorectal tissue at higher percentages when compared to penile glans tissue ($P=0.037$) and whole blood ($P=0.029$) (Figure 3.7A).

Percentages of NK cells isolated from penile glans tissue were higher when compared to cells isolated from all other tissue types and whole blood,

although this trend only reached significance when compared to NK cells isolated from colorectal tissue (P=0.011) (Figure 3.7A.).

The most abundant cell subset isolated from whole blood were the neutrophils (CD3-CD15+CD66b+), representing approximately 80% of all viable cells. There were significantly higher percentages of neutrophils isolated from whole blood when compared to penile glans (P=0.035) and ectocervical tissue (P=0.011) (Figure 3.7A), with a trend for higher levels when compared to colorectal tissue. Importantly, in the context of this investigation, it should be noted that neutrophils were found infrequently within all three mucosal tissues. This low expression meant that too few cell numbers were acquired to perform meaningful FcR expression analysis, so this cell subset is not represented in the FcR profile analysis.

Combined phenotypic analysis of the three cell subsets of interest for further in depth investigation into FcR expression (CD14+ monocytic cells, mDC and NK cells) show that, while there is a trend for higher expression of CD14+ monocytic cells isolated from colorectal tissue and NK cells isolated from penile glans tissue, these differences do not reach statistical significance in most instances when compared to the other tissue types (Figure 3.7).

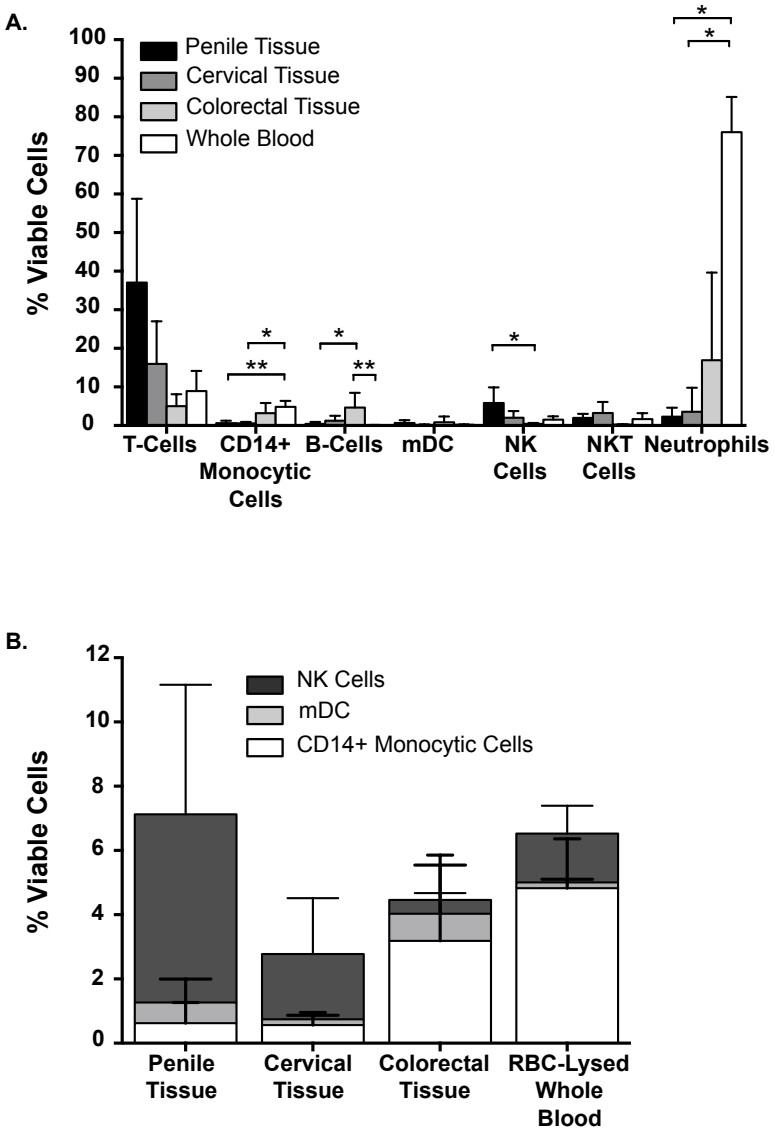


Figure 3.7 Overall immune cell phenotypes found within penile glans, ectocervical and colorectal tissue and RBC-lysed whole blood.

The percentage of CD3+ T cells, CD14+ monocytic cells, CD19+ B cells, myeloid DCs (mDC), NK cells, NKT cells and neutrophils in total viable cells isolated from (A) penile glans (n=9), ectocervical (n=5) and colorectal (n=6) tissue and RBC-lysed whole blood (n=6). (B) Relative proportions of the cells of interest for FcR expression. (Mean and SD values shown in both graphs). Statistical analysis performed using Kruskal-Wallis with Dunn's multiple comparison test. [*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001]

3.2.4 CD14+ Monocytic Cell FcR Expression in Whole Blood and Mucosal Tissue Compartments

CD14 is a surface marker expressed by monocytic cells, mainly macrophages and DCs. These cells are capable of killing pathogens through ADCC and ADCP. Expression of FcR (CD16, CD32, CD64 and CD89) on monocytic cell populations isolated from mucosal tissues or whole blood was determined using the gating strategy outlined in Figure 3.4A.

CD14+ monocytic cells isolated from whole blood expressed higher levels of all FcR stained for when compared to mucosal tissues. CD16 was found at significantly higher levels when compared to penile tissue (P=0.005) (Figure 3.8A). CD32 was found at higher levels when compared to penile (P=0.005) and colorectal (P=0.007) tissue, however the lower levels of expression noted on cells isolated from ectocervical tissue did not reach significance. Greater levels of CD64 and CD89 were observed when compared to penile glans (P=0.007 & P=0.014, respectively), ectocervical (P=0.022 & P=0.038, respectively) and colorectal tissue (P=0.028 & P=0.008, respectively) (Figure 3.8A).

Boolean gating, a method of analysing large flow cytometry panels by producing data on all possible combinations of selected markers, was applied to investigate the combinatorial nature of FcR expression on CD14+ monocytic cells. Interestingly, approximately half of all viable CD14+ monocytic cells isolated from the three mucosal tissues were found to be negative for the FcR investigated (Figure 3.8B & C). These data are in contrast to the expression pattern seen in whole blood, which revealed co-expression of CD32, CD64 and CD89 in around 65% of CD14+ monocytic cells (Figure 3.8B & C). This observation could be caused by downregulation of FcR at the mucosal sites to

avoid over-stimulation of the effector cells within the mucosal environment and/or reflect terminal differentiation.

When investigating the cells that were positive for FcR in all mucosal tissues, higher expression levels of receptors associated with ADCP and ADCVI (CD32, CD64 and CD89) were observed when compared to ADCC (CD16). Although the level of FcR expression between the three mucosal tissues is statistically comparable, it is important to note that there were approximately 6-fold more CD14+ monocytic cells found in colorectal tissue when compared to penile glans and ectocervical tissue (Figure 3.7B). This observation potentially indicates that, while FcR expression patterns do not differ significantly between mucosal tissue types, there could be a variation in the absolute number of cells available to elicit downstream effector functions.

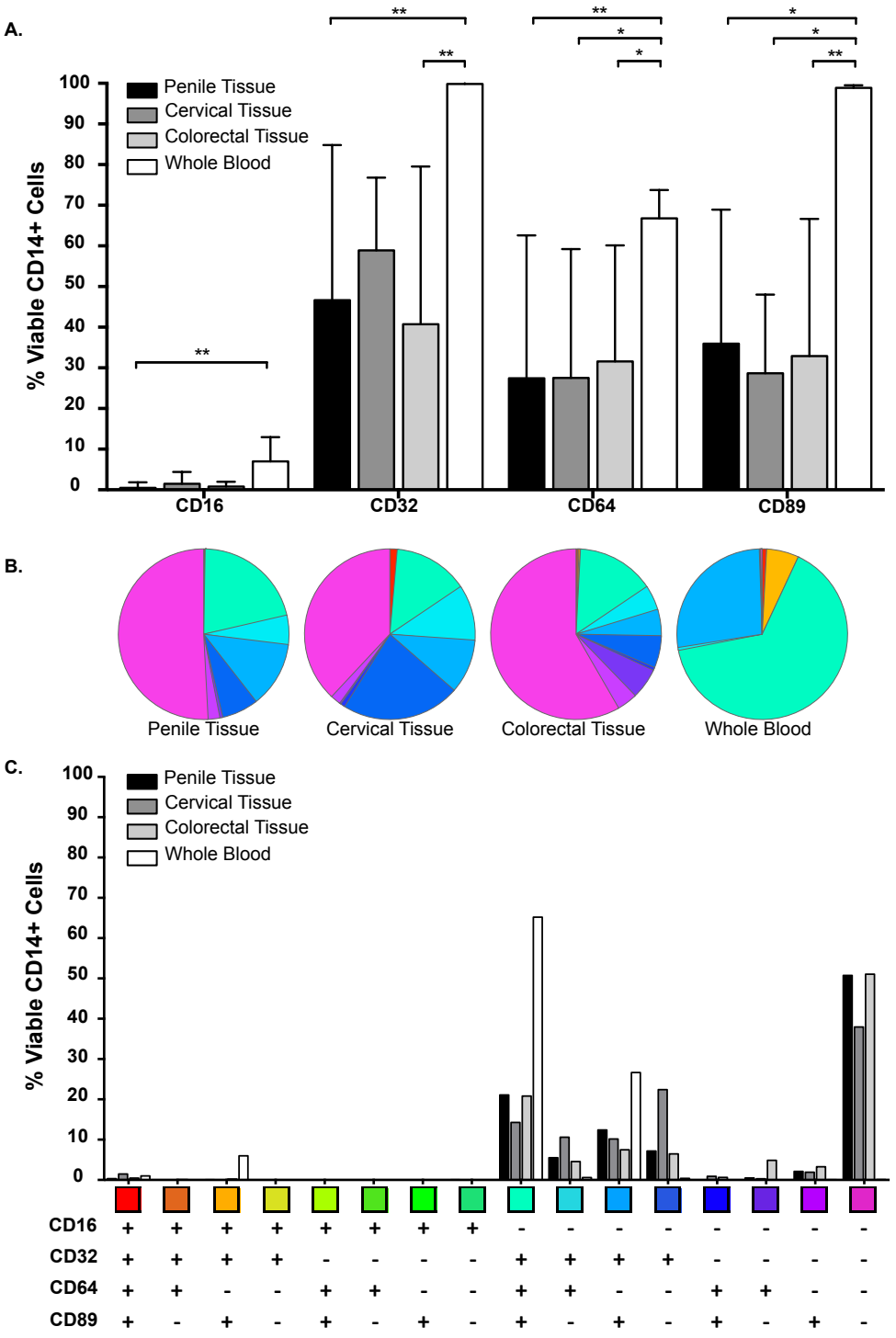


Figure 3.8 Fc Receptor expression on CD14+ monocytic cells isolated from penile glans, ectocervical and colorectal tissue and RBC-lysed whole blood.

(A) Percentage expression of CD16, CD32, CD64 and CD89 on viable CD14+ monocytic cells (Mean and SD values shown; Statistical analysis performed using Kruskal-Wallis with Dunn's multiple comparison tests]. (B) and (C) Boolean gating analysis to show the combinatorial nature of FcR expression in CD14+ monocytic cells isolated from mucosal tissue and whole blood [Mean values shown]. [*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001]

3.2.5 FcR Expression on Myeloid DCs

Myeloid DCs (mDC) are primarily involved in antigen presentation; however, they are also capable of phagocytosis of infectious pathogens through engagement of FcR. Expression of FcR on the surface of mDC isolated from the mucosal tissues or whole blood was investigated, with mDC defined as any viable cells that were CD3-, $CD14^-$, $CD19^-$ and $CD11c^+$ (Figure 3.4A).

The most prevalent FcR detected on mDC isolated from mucosal cells and whole blood was CD32. Significantly higher levels of CD32 were found on mDC isolated from whole blood when compared to colorectal (P=0.036) and penile glans (P=0.012) tissue. mDC isolated from whole blood demonstrated higher levels of CD16 when compared to all mucosal tissue types, reaching significance when compared to ectocervical (P=0.004) and penile glans (P=0.002) tissue. A trend was observed for greater levels of CD64 on the surface of mDC isolated from all three mucosal tissues when compared to whole blood, although this difference did not reach significance. All compartments demonstrated similar levels of CD89 (Figure 3.9A)

When combinatorial Boolean gating was used to investigate the complexity of FcR expression, a greater diversity of FcR expression was observed on mDC isolated from mucosal tissues. Indeed, mDC isolated from whole blood demonstrated almost exclusive expression of CD32, which was expressed on approximately 90%, (Figure 3.9B & C). As was observed for CD14+ monocytic cells, a greater proportion of mDC isolated from mucosal tissue did not express any FcR when compared to the whole blood. However, the mDC that were positive for FcR were more diverse in their expression (Figure 3.9B & C). Although no significant difference was noted between the percentages of mDC

found across the three mucosal tissues, a trend for increased percentages in penile glans and colorectal tissue was observed (Figure 3.7B).

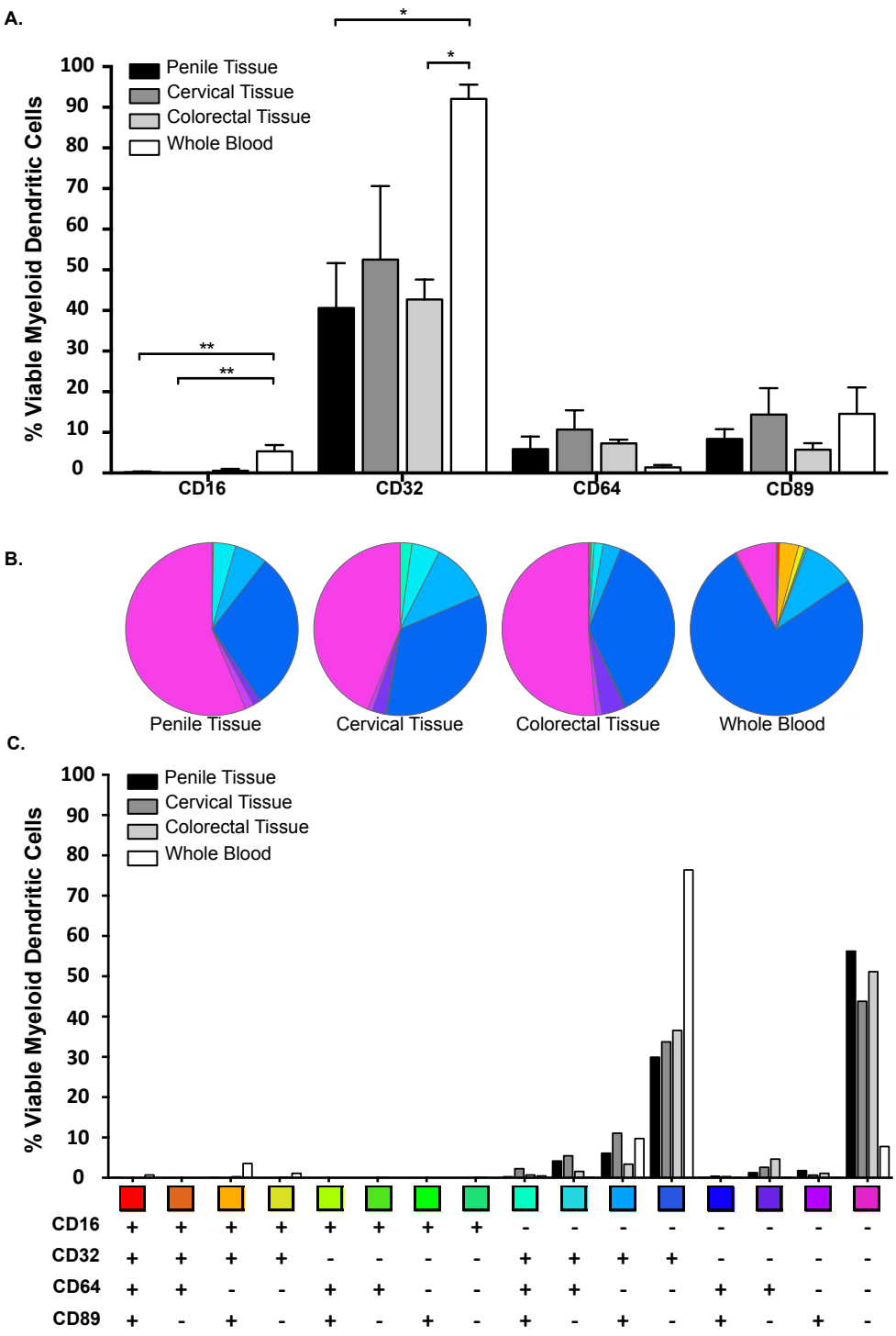


Figure 3.9 Fc Receptor Expression on Myeloid DCs isolated from penile glans, ectocervical and colorectal tissue and RBC-lysed whole blood.

(A) Percentage expression of CD16, CD32, CD64 and CD89 on viable mDC isolated from mucosal tissues and whole blood [Mean and SD values shown. Statistical analysis performed using Kruskal-Wallis with Dunn's multiple comparison tests]. (B) and (C) show Boolean gating to show the combinations of FcR expression across the isolated mDC [Mean values shown]. [*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001]

3.2.6 NK Cell FcR Expression in Whole Blood and Mucosal Tissue Compartments

NK cells are innate cytotoxic cells that are primarily involved in the killing of virally infected cells through release of cytokines during ADCC. FcR, particularly CD16, play an important role in enabling the gap to be bridged between these innate cells and antibodies of the humoral immune system. Here, the gating strategy outlined in Figure 3.4B was used to determine the FcR on the surface of NK cells, which were defined as any viable cells that were $CD3^-$, $CD56^+$.

CD16 was the most prevalent FcR detected on NK cells isolated from ectocervical and penile glans tissue and whole blood. While fewer CD16+ NK cells were isolated from colorectal tissue when compared to the other compartments, this variability was only significant when compared to NK cells isolated from whole blood (P=0.033). Similar levels of CD32, CD64 and CD89 were observed on NK cells isolated from ectocervical and colorectal tissue and whole blood, with lower levels noted on penile glans tissue. However, this difference did not reach significance (Figure 3.10A).

Once again, Boolean gating analysis was applied to assess the combinatorial expression of FcR on NK cells. As observed for monocytic CD14+ cells and mDC, there were more FcR negative NK cells isolated from mucosal tissues when compared to whole blood, with the phenotypic difference most evident for NK cells isolated from colorectal tissue, which included approximately 65% FcR negative cells. The most diverse FcR expression was noted in NK cells isolated from ectocervical tissue (Figure 3.10B & C).

A trend for a higher percentage of NK cells was noted in the penile glans tissue when compared to all other compartments, reaching significance when

compared to the colorectal tissue (Figure 3.7B). Although this increased percentage does not inform on absolute numbers of NK cells present, it could impact on the number of FcR and effector cells available for further downstream functions.

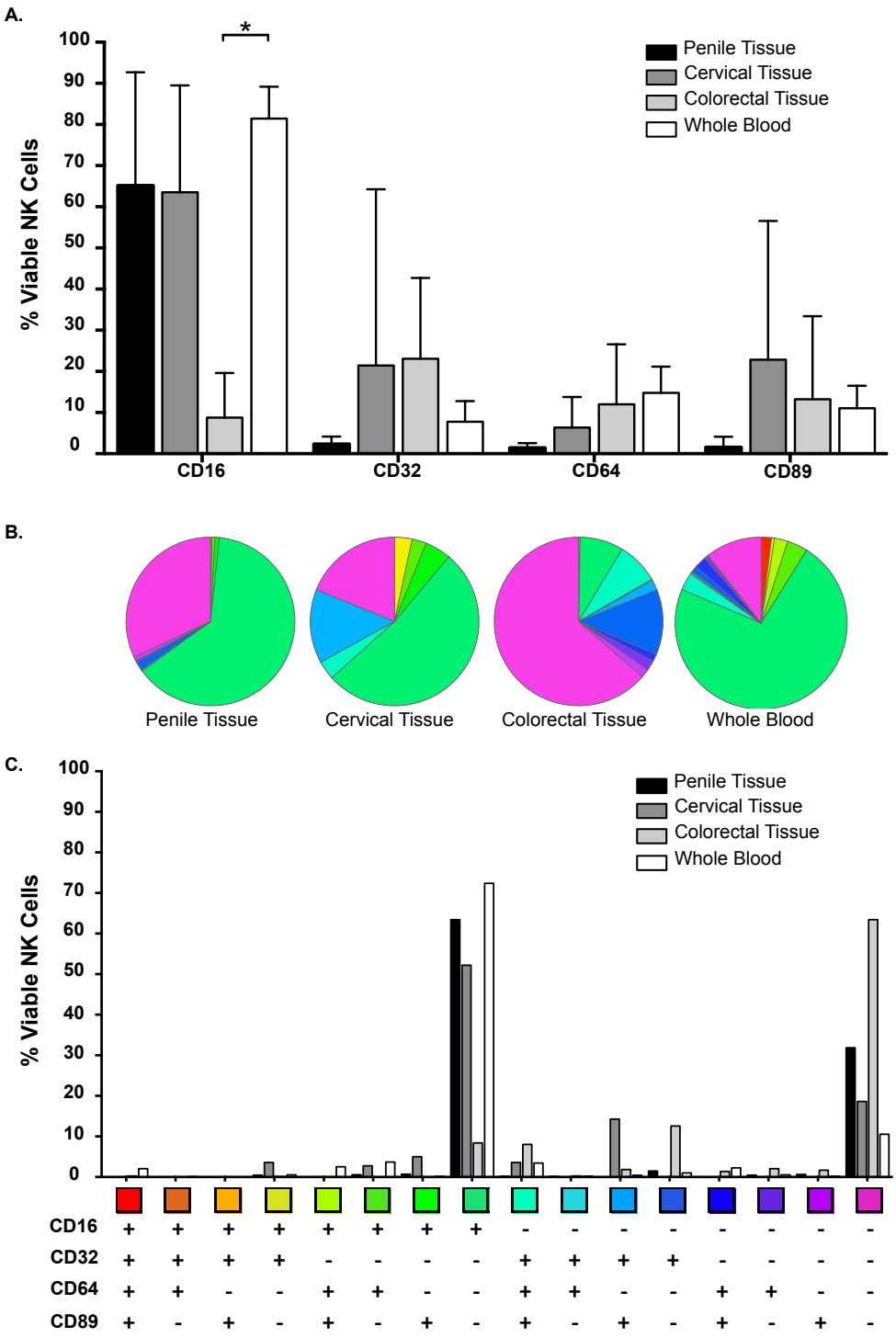


Figure 3.10 Fc Receptor expression on NK cells isolated from penile glans, ectocervical and colorectal tissue and RBC-lysed whole blood.

(A) Overall percentage expression of CD16, CD32, CD64 and CD89 on viable NK cells isolated from mucosal tissue and whole blood [mean and SD values shown. Statistical comparisons were made using Kruskal-Wallis with Dunn's multiple comparison tests]. (B) and (C) show the Boolean gating analysis, demonstrating the combinations of FcR expression found on NK cells isolated from the mucosal tissues or whole blood. [*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001]

3.2.7 Location of Immune Effector Cells and FcR within Mucosal Tissue.

To inform on the potential availability of immune effector cells to interact with antibody and initiate further downstream effector functions within mucosal tissues, deconvolution microscopy was used to determine the location of CD14+, CD11c+ or CD56+ cells. Secondary staining was used to further assess FcR location and co-localisation within the tissues.

To assist with this work, samples of tissue were frozen within our laboratory and sent to the laboratory of Thomas J. Hope, Northwestern University, Chicago, USA. In collaboration, the staining antibodies were selected to determine CD14, CD11c, CD56 and FcR (CD16, CD32, CD64 and CD89) expression. Sections were prepared and stained by Ann M. Carias at the laboratory in Chicago, with final images being sent back to our laboratory for analysis and presentation purposes.

Within penile glans tissue, CD14+ were almost exclusively located in the subepithelial lamina propria of the squamous epithelium. In one, rare instance, a CD14+ cell was noted within the stratum basale (Figure 3.11A). CD14+ cells were found to reside within the subepithelium and stratum basale of ectocervical tissue (Figure 3.11B) while, within the colorectal tissue, CD14+ cells were exclusively found within the lamina propria (Figure 3.11C).

The subepithelial lamina propria of the penile tissue was the exclusive location for CD11c+ cells (Figure 3.12A), while they were more diffusely located throughout the squamous epithelium of the ectocervical tissue, from the stratum spinosum into the stratum basale and deeper into the subepithelium (Figure 3.12B). The lamina propria of the colorectal tissue was the only location that CD11c+ cells were noted (Figure 3.12C).

The most diffusely located effector cell type stained for within penile tissue were the CD56+ cells, with the majority residing within the subepithelial lamina propria, However, CD56+ cells were also observed within the stratum basale and, on one occasion, a CD56+ cell was noted near the luminal border (Figure 3.13A). Within ectocervical tissue, CD56+ cells were primarily observed within the subepithelium or stratum basale (Figure 3.13B), whereas CD56+ cells of the colorectal tissue were, once again, found to be exclusively located within the lamina propria (Figure 3.13C). Co-localisation of the effector cells and FcR were in line with the flow cytometry data in all mucosal tissue types investigated (Figures 3.11-3.13).

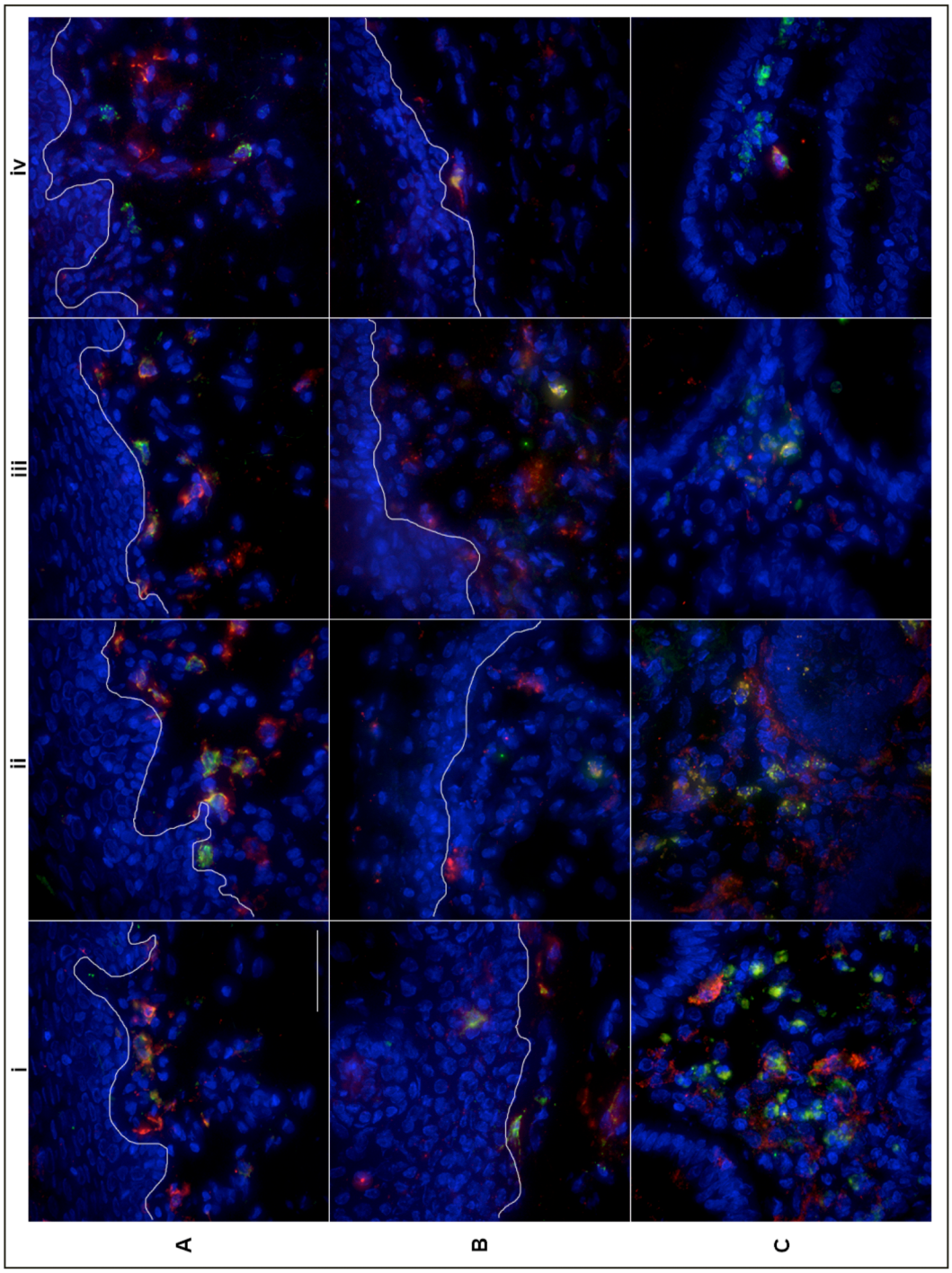


Figure 3.11 Location of CD14+ cells within mucosal tissues.

Deconvolution fluorescent microscopy of (A) penile glans, (B) ectocervical and (C) colorectal tissue to show CD14+ cellular location (green) and relative co-localisation of (i) CD16, (ii) CD32, (iii) CD64 and (iv) CD89 (all red). DAPI staining is shown in blue. Images taken at 40x magnification. Scale bar is set to 40 microns. All sectioning, staining and imaging performed by Ann Carias, Northwestern University, Chicago, USA.

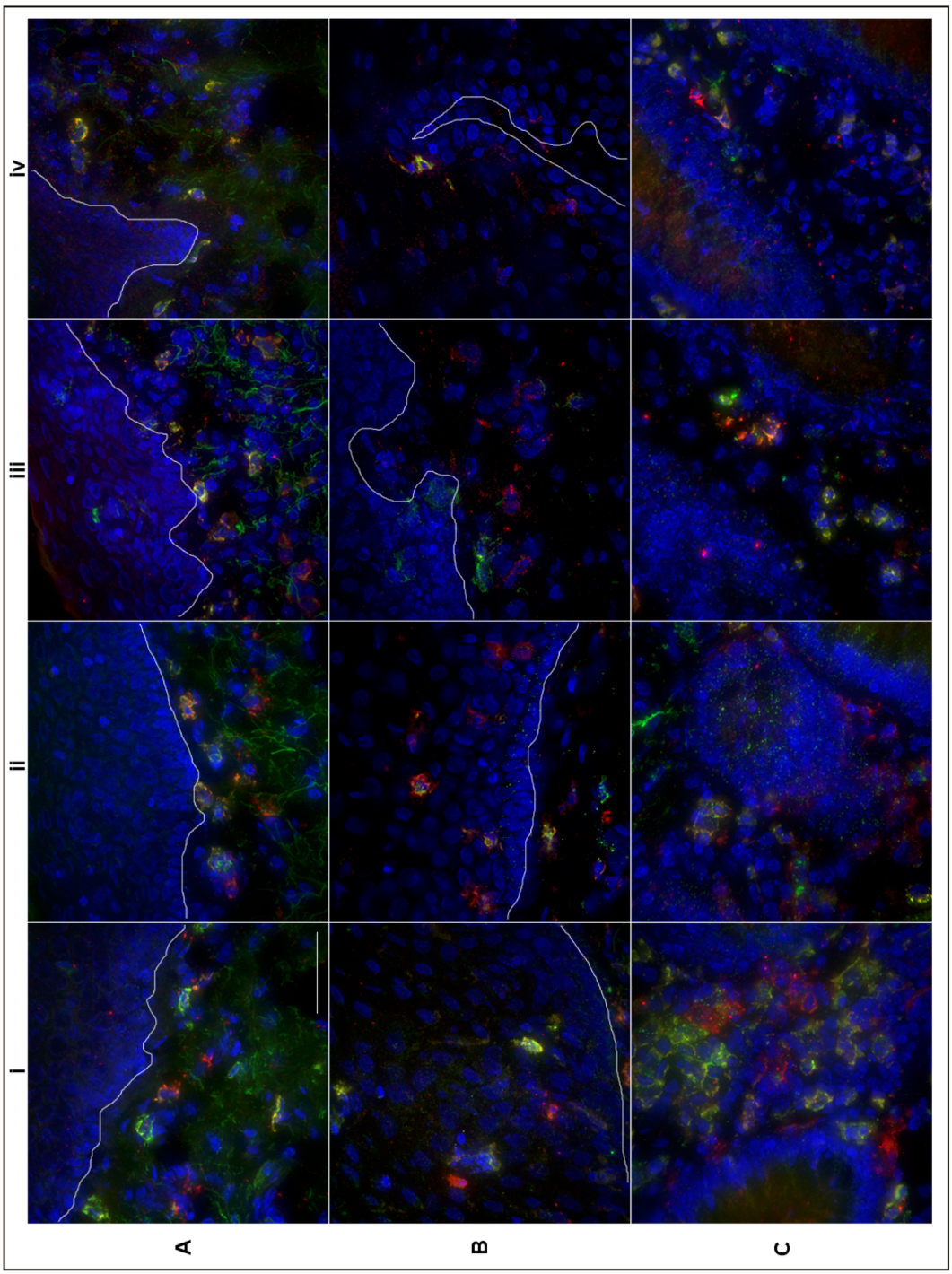


Figure 3.12 Location of CD11c+ cells within mucosal tissues.

Deconvolution fluorescent microscopy of (A) penile glans, (B) ectocervical and (C) colorectal tissue to show CD11c cellular location (green) and relative co-localisation of (i) CD16, (ii) CD32, (iii) CD64 and (iv) CD89 (all red). DAPI staining is shown in blue. Images taken at 40x magnification. Scale bar is set to 40 microns. All sectioning, staining and imaging performed by Ann Carias, Northwestern University, Chicago, USA.

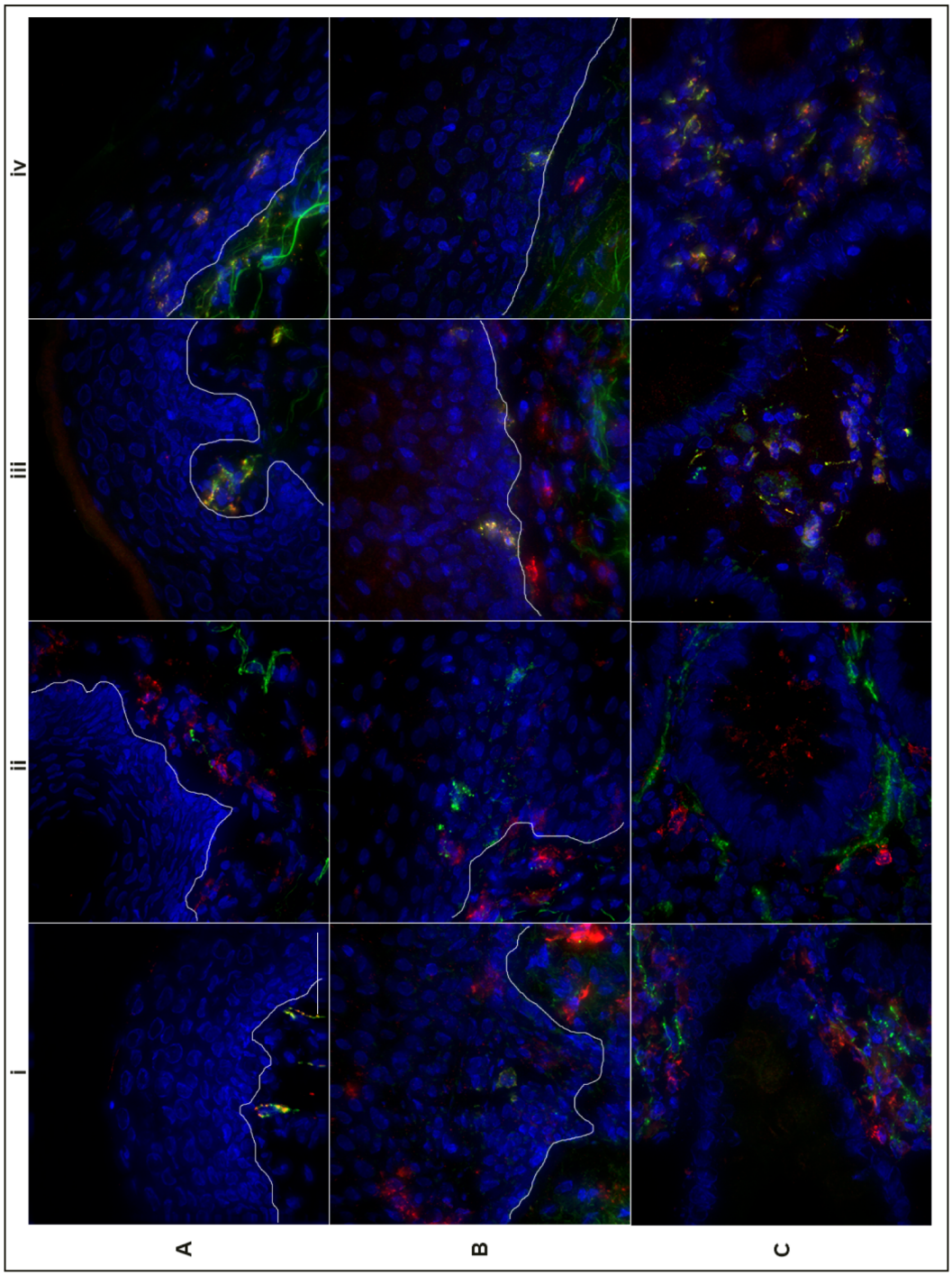


Figure 3.13 Location of CD56+ cells within mucosal tissues.

Deconvolution fluorescent microscopy of (A) penile glans, (B) ectocervical and (C) colorectal tissue to show CD56 cellular location (green) and relative co-localisation of (i) CD16, (ii) CD32, (iii) CD64 and (iv) CD89 (all red). DAPI staining is shown in blue. Images taken at 40x magnification. Scale bar is set to 40 microns. All sectioning, staining and imaging performed by Ann Carias, Northwestern University, Chicago, USA.

3.3 Discussion

This chapter set out to characterise the relative FcR expression levels and composition of FcR positive cells within male and female genital mucosal tissue, colorectal tissue and RBC-lysed whole blood with the goal that this would help better understand the potential of antibody-dependent cellular effector functions to impact on early HIV-1 transmission events at mucosal surfaces.

Firstly, it was important to determine the cellular phenotypes found within penile glans, ectocervical and colorectal tissue and whole blood before further investigating the effector cell subsets for FcR expression. Similar percentages of CD3+ T cells, NKT cells and mDC were observed across the three tissue types and whole blood. However, there were significantly different percentages of CD14+ monocytic cells, CD19+ cells and neutrophils observed between the mucosal tissues and whole blood.

Colorectal tissue displayed higher percentages of CD19+ cells when compared to ectocervical and penile glans tissue, which is reflective of previous studies that have shown high CD19+ cells within the lower gastrointestinal tract [396, 397]. Furthermore, the percentage of CD14+ monocytic cells isolated from the colorectal tissue was higher when compared to penile glans or ectocervical tissue, albeit at lower levels than those isolated from whole blood. Previous investigations into CD14 expression within intestinal tissue have revealed some conflicting data. While low-level CD14 expression has previously been observed on macrophages isolated from colonic tissue [145, 398], it is important to note that these studies used pure macrophage populations, whereas this study investigates pan-CD14+ monocytic cells isolated from colorectal tissue, with no subsequent markers of macrophage phenotype. More reflective of the data

observed in this chapter are results from a recent study using confocal microscopy to investigate CD14+ cells within the sigmoid colon and rectal mucosa [399]. Furthermore, the higher frequency of colorectal CD14+ monocytic cells observed here when compared to the penile glans or ectocervical tissues could be attributed to an influx of monocytes from the periphery, as tissue-resident macrophages of the gastrointestinal tract are almost exclusively derived from the recruitment of blood monocytes [400, 401].

Neutrophils are reported to be the most common leucocyte subset within whole blood, which is reflected in the data observed in this chapter [402]. When investigating neutrophils isolated from the mucosal tissues, it was not possible to perform an accurate analysis of FcR expression due to low recovery of this cell type. This is similar to the results of others who observed low neutrophil numbers within the skin (penile glans) [403], ectocervical [404] and colorectal tissue [405]. However, given that neutrophils constitutively express high levels of CD16B and CD89 and low levels of CD16A and CD32, and are one of the first cell subsets recruited during inflammation, it is possible that they may still provide a functional role in mucosal HIV-1 immunity [406]. Furthermore, neutrophils can be induced to express CD64 by IFN-γ leading to increased ADCC activity [153], which may be of relevance in an inflammatory environment such as that induced during the early stages of HIV-1 infection.

When investigating the location of immune effector cells (CD14+, CD11c+ and CD56+), the results reflect previous studies demonstrating that these cell subsets tend to reside in the subepithelial and basal layers of mucosal tissue [265, 297, 397, 407]. The fact that these cells are not found superficially within

the tissue could be important when considering their availability to interact with vaccine-induced HIV-1 antibodies and prevent early viral transmission events.

The second aim of this chapter was to determine the FcR expression on the surface of CD14+ monocytic cells, mDC and NK cells. The effector functions induced by Fc-FcR engagement depend upon the type of FcR activated and the effector cell subset the FcR is expressed by. Most commonly associated with ADCC activity through NK cells, CD16 is the low/intermediate affinity receptor for IgG. Effector functions include release of lytic vesicles containing perforin and granzyme, induction of phagocytosis of immune complexes and cytokine release. As the low affinity IgG FcR, CD32 requires antibodies to have formed immune complexes with the antigen, inducing crosslinking of the FcR, to trigger further downstream effects such as target cell lysis and/or phagocytosis. The high affinity IgG FcR, CD64, also induces phagocytosis and has particular affinity for IgG1 and IgG3 [146]. Finally, in the context of this thesis, CD89 (FcαR), the IgA receptor, binds aggregated IgA complexed with antigen leading to phagocytosis and ADCC [100]. Furthermore, in addition to antibody-mediated effector functions, FcγR have a regulatory role in DC activity and are important in determining whether an inflammatory or tolerogenic response is initiated during antigen presentation from DCs to T cells. [130].

High levels of CD16 were observed on NK cells isolated from penile glans and ectocervical tissues and whole blood, while levels were low on NK cells isolated from colorectal tissue. This is reflective of a previous study that found NK cells within normal colorectal tissue to be of a CD56+ $CD16^{lo/-}$ phenotype [408]. Furthermore, on CD14+ monocytic cells, CD16 expression was comparatively low when compared to CD32, CD64 and CD89, receptors more

commonly associated with ADCP and ADCVI. This possibly indicates that ADCP and ADCVI might have increased potential over ADCC for impacting on HIV-1 transmission events. While it is not possible to determine whether the CD14+ monocytic cells investigated are macrophages, recent work by another group has demonstrated that macrophages can be isolated from colonic and cervical mucosal tissue. Indeed, this group further explored the phagocytic activity of tissue-resident macrophages and showed them to have a greater propensity for antibody-mediated activity when compared to tissue-resident NK cells, potentially indicating a role for these cell types in protection from infection [174].

It should be observed that the CD16 monoclonal antibody used throughout this study was not capable of distinguishing between CD16A and CD16B receptors. However, previous studies have demonstrated that CD16B is exclusively found on the surface of neutrophils, while CD16A is found on the surface of NK cells, DCs and macrophages [409, 410]. These studies lend weight to the summaries made throughout this chapter that the CD16 expression observed on CD14+ monocytic cells, DCs and NK cells is CD16A.

The most commonly observed FcR here was CD32, which was found predominantly on CD14+ monocytic cells and mDCs, irrespective of tissue type. This is unsurprising, given that CD32 has been shown to be the most broadly expressed FcγR on immune effector cells found in the blood [147]. However, a caveat of this study is that it was not designed to distinguish between inhibitory (CD32B) or activating (CD32A or CD32C) forms of CD32. Inhibitory receptors are found on the surface of B cells, as well as some subsets of macrophages, DCs and granulocytes. The regulatory function of the inhibitory receptor is

controlled by the presence of an immunoreceptor tyrosine-based inhibitory motif (ITIM) transmembrane domain [411]. CD32B on the surface of DCs is important in enforcing immune tolerance by preventing T cell responses [412]. Activating FcR signal through the immunoreceptor tyrosine-based activating motif (ITAM) transmembrane domain, with the ultimate outcome of receptor activation being antigen processing and presentation or initiation of further downstream effector functions, such as ADCC, ADCP or ADCVI. In the context of HIV-1, antibodies isolated from HIV-1 infected individuals not on therapy have been shown to have skewed interactions with CD32A and CD32B, preferentially binding to CD32A. The antibodies were deemed to have altered Fc domain glycosylation in both total plasma and HIV-specific IgG, leading to increased phagocytic activity [151]. Furthermore, CD16 and CD32 receptors are subject to allelic variation, which this study was not designed to investigate. However, it is important to observe that polymorphic variants of activating receptors CD16A and CD32A can affect their ability to bind to IgG subclasses. CD16A can include two variants with polymorphisms at position 158, leading to variants FcγRIIIa$_{V158}$ and FcγRIIIa$_{F158}$. Similarly, allelic variants within CD32A are caused by polymorphisms at position 131, leading to two variants: FcγRIIa$_{R131}$ and FcγRIIa$_{H131}$. All allelic variants bind equally to IgG1 and IgG3, but FcγRIIa$_{H131}$ binds with higher affinity to IgG2 when compared to FcγRIIa$_{R131}$ and FcγRIIIa$_{V158}$. IgG4 binds with high affinity to all FcR, with the exception of FcγRIIIa$_{F158}$, which binds at a lower affinity [133]. Allelic variants have been proposed as another correlate of protection in the RV144 trial [135, 136]. The ability to distinguish between the CD32 receptor phenotypes and allelic variants within individuals

and across different mucosal tissue types would be important to fully characterise in future studies.

CD64 was found at significantly lower levels on the surface of CD14+ monocytic cells isolated from all three mucosal tissues when compared with freshly isolated whole blood. Loss of CD64 expression is a key early indicator of myeloid cell maturation [413]. It is therefore possible that this observation is indicative of mucosal CD14+ cells being of a more mature phenotype than those found within whole blood. If this is found to be the case, it could be of relevance when considering the ease of inducing CD64-specific effector immune responses at mucosal surfaces through vaccination.

Regardless of the source, low-level CD89 surface expression was observed on NK cells, while similar levels were found on the surface of CD14+ monocytic cells and mDC isolated from mucosal tissue. However, reflective of previous studies, CD89 expression on the surface of tissue-isolated CD14+ monocytic cells was significantly lower than that found on cells isolated from whole blood [160].

The expression of FcR is controlled by a number of factors. For example, cytokines such as IL-4 and IFN-γ can upregulate the expression of FcγR on monocytes and neutrophils [414, 415], while TNF-α expression upregulates FcγR and CD89 expression on NK cells [158, 159]. Furthermore, IL-10 has been shown to downregulate FcγR expression on monocytes [416]. Within a mucosal environment such as the gastrointestinal tract, cytokine levels and production can be affected by factors such as diet, pre/probiotics and antibiotics [417, 418]. Furthermore, within the female reproductive tract cyclical hormonal fluctuations can also change the cytokine environment [266, 267]. While within

penile tissue, cytokine production differs between inner & outer foreskin and the penile glans [301], with separate studies investigating cytokine profiles in penile foreskin in healthy and HIV-1 infected individuals [295, 296, 419]. For penile glans, studies investigating cytokine profiles within the skin may also be considered, with results indicating differences in profiles observed between the skin and periphery [420]. Therefore, it should not be discounted that the lower FcR expression levels observed within the three mucosal tissues are as a result of the different local environments from which they were isolated.

A further caveat is that all mucosal tissues used in this chapter were obtained from patients attending clinics in London, UK. There is potential for FcR expression profiles to vary in individuals from different countries throughout the world, where external influences could alter FcR expression. For example, it has been shown that *Plasmodium falciparum* infection induces upregulation of FcγR on monocytes and subsets of DC [421, 422]. Given that a large number of HIV-1 vaccine trials are conducted in malaria endemic regions, it would be important to further characterise the FcR expression in mucosal tissue from individuals from these regions.

Overall, this chapter sought to characterise the pattern of FcR expression on CD14+ monocytic cells, mDC and NK cells across three different mucosal tissues associated with HIV-1 transmission. To summarise, the data showed:

- FcR expression levels were significantly lower on effector cells isolated from mucosal tissues than whole blood.
- Immune effector cells were predominantly located deeper within healthy mucosal tissue.

- A large proportion of mucosal myeloid cells were negative for all FcR.
- Mucosal myeloid cells positive for FcR displayed a greater diversity of FcR expression than those in the periphery

Together, the results show that immune cells of mucosal tissue are phenotypically different when compared to those found in the periphery, which may have important implications for the potential of antibody-dependent cellular effector mechanisms to impact on HIV-1 transmission events. To date, most *in vitro* ADCC assays use blood-derived NK cells, neutrophils, monocytes or macrophages. While blood-derived monocytes mediate similar levels of ADCC to NK cells [423], it is unclear as to the relative potency of their mucosal counterparts. Indeed, it may be that other antibody-mediated functions such as ADCP, play a greater role in HIV-1 protection in mucosal tissues [174].

Experiments conducted in Chapter 4 further investigate the relative activity of nAb and nnAb within mucosal explant models and will help to determine whether antibody-mediated effector functions are enough to prevent infection within mucosal tissues.

CHAPTER 4 – RESULTS:

Functional Assessment of Antibody Activity in Cellular and Mucosal Tissue Explant Inhibition Assays

4. Functional Assessment of Antibody Activity in Cellular and Mucosal Tissue Explant Inhibition Assays

4.1. Introduction

During the natural course of HIV-1 infection, the development of effective immune responses varies between individuals. BnAb, capable of neutralising infection across multiple viral clades, are approximated to occur in around 10% of infected individuals. Furthermore, nAb, capable of neutralising clade-specific virus, are thought to occur in approximately 50% of HIV-1 infected individuals [424]. While both bnAb and nAb require several years and many rounds of SHM before developing [425], nnAb are found in all HIV-1 infected individuals from the acute stage of infection onwards [426].

Classical neutralisation of HIV-1 infection by nAb is triggered by the binding of the antibody to the trimeric envelope spike, blocking key epitopes on the surface of the virus, inhibiting engagement with cell receptors and thus preventing viral fusion and entry [219]. Amongst others, nAb have been found against the CD4bs, gp41 MPER, and glycan-dependent epitopes on gp120. However, it is important to note that there is some diversity in the HIV-1 envelope structure, leading to a complex mix of functional trimers and non-functional dimers, monomers and gp41 stumps that have shed all of their gp120 [34]. Antibodies developing against these various non-functional forms of the envelope are more likely to be non-neutralising [220].

For nnAb to confer protection, the Fab portion of the antibody must first recognise and bind to an epitope on the surface of a pathogen, coating it with antibody. Interaction of the opsonised pathogens with FcR leads to activation of

effector cells and further downstream activities, such as ADCP, ADCC or ADCVI. It is also important to remember that antigen can be opsonised by complement that initiates an immune response when it interacts with the CR1 receptor on the surface of innate immune effector cells [125, 427]. However, given the scope and purpose of this project to investigate Fc-mediated antiviral function, complement activity will not be studied.

Published data has demonstrated that FcR are also important for the activity of nAb. A study in macaques demonstrated that, by mutating the Fc-region of the nAb, a reduced ability to prevent Simian-Human Immunodeficiency Virus (SHIV) infection was observed, although the inhibitory ability of nAb was not completely abolished [385]. This data has been used to highlight the potential importance of Fc function in nAb and to show the potential relevance of FcR effector function in nnAb-derived inhibition of HIV-1.

The only vaccine trial to date to show any protective efficacy, the RV144 trial, did so in the absence of bnAb or tier 2 virus neutralising ability [353]. Extensive correlate of protection analysis identified that there was a positive association with high levels of serum IgG antibodies to the V1V2 region of Env capable of ADCC [134, 354, 356]. It is important to note that this positive association was reduced in the presence of high levels of IgA antibodies, capable of competitively binding to the same V1V2 region [356]. The discovery that the protection conferred by the vaccine was linked to nnAb capable of ADCC combined with the challenges of inducing bnAb has boosted the idea that nnAb might constitute an important role in the development of a prophylactic vaccine [354, 355, 358, 374, 375, 383, 384]. Although it is important to note that it is currently unclear if this is a mechanistic correlate or a surrogate of another

immune response.

Several passive immunisation studies using nAb and nnAb in macaques have shown that topical application of nAb was effective at preventing SHIV infection [166, 168]. Further passive infusion studies noted that, while nnAb were not capable of preventing infection, a lower viral set-point was achieved and the number of founder viruses were more limited after administration of nnAb [240, 428]. These findings indicate that antibody-FcR interactions play an important role at the initial stages of infection and their role in subsequent disease progression should not be discounted. Indeed, studies of HIV-1 positive individuals have indicated that the presence of nnAb capable of ADCC correlate with slower disease progression [167, 170], give rise to significantly lower genital HIV-1 RNA levels in seropositive women [171] and, when found in breast milk, reduce the risk of MTCT [172]. Further investigations into the functional activities of nnAb in HIV-1 positive long-term slow-progressors (LTSP) have identified that ADCC-inducing antibodies in these individuals exhibit wider breadth of antibody reactivity and greater magnitude of response [429, 430]. In addition, glycosylation of the Fc-domain of an antibody can modulate how an antibody interacts with the FcγR. Agalactosylated antibodies have a greatly augmented ability to bind CD16, leading to enhanced effector functions. Increased frequencies of agalactosylated antibodies have been documented in HIV-1 controllers [241].

During the initial stages of HIV-1 transmission at mucosal surfaces, antigen-presenting cells such as DCs and macrophages, play a role in the uptake and subsequent presentation of HIV-1 to CD4+ target cells [431-434]. DCs transfer HIV-1 infection to T cells via one of two routes: *Cis*-infection, where *de*

novo infection of DC subsequently transmits the virus to effector cells, and *trans*-infection [67], where DC do not become productively infected, but instead infect CD4+ cells via an infectious synapse mediated by DC-SIGN-CD4 T cell receptor interaction or by endocytosis and release of the virus containing exosomes. LC, a subset of DCs, have been found in the skin, oral and genital mucosa, but not in the intestinal mucosa [73-75]. These cells express Langerin (CD207), a C-type lectin that captures antigen and presents it to T cells. Langerin has been shown to bind gp120 on the surface of HIV-1, limiting infection by internalising the virus and degrading it within Birbeck granules [76, 77]. If HIV-1 has been opsonised by IgG nnAb, Langerin cannot bind the gp120 on the virus and the antiviral protection it provides is no longer active [83, 435]. However, this is a complex picture, with other research demonstrating that nnAb are capable of inhibiting infection, *in vitro* [436]. In addition, a study in South Africa showing that circumcised men had a greatly reduced risk of acquiring HIV-1, which is thought due to the lack of foreskin and therefore lower numbers of LC at site of potential transmission [290].

While both macrophages and DCs are capable of becoming productively infected by HIV-1 [68], importantly, they are also both capable of initiating antibody-dependent effector functions through FcR-engagement [437]. In Chapter 3, the FcR expression and effector cell profiles of tissues associated with mucosal HIV-1 transmission were investigated. Results demonstrated that FcR and effector cells (CD14+ monocytic cells, mDCs and NK cells) were all present in mucosal tissues, albeit at lower levels than RBC-lysed whole blood. However, the potential for these cells to exert an inhibitory effector function when in the presence of HIV-1 specific antibodies remains to be determined.

In this chapter, the role of antibody Fc-mediated antiviral activity will be assessed in different tissue and cellular models designed to mimic the initial events required to establish mucosal infection. A panel of nAb and nnAb, including those associated with protection in the RV144 vaccine trial, will be developed and assessed for antiviral activity within the inhibition models.

The cellular inhibition assays use monocyte-derived macrophages (MDM), monocyte-derived DCs (MDDC) or peripheral blood mononuclear cells (PBMC) to determine the ability of antibodies to inhibit direct infection of these cells. Expanding on the concept that DCs are capable of promoting onward HIV-1 transmission to CD4+ T cells, the DC-T cell model assesses the ability of the antibodies to block *trans*-infection from DC-to-CD4+ T cells (Figure 4.1)

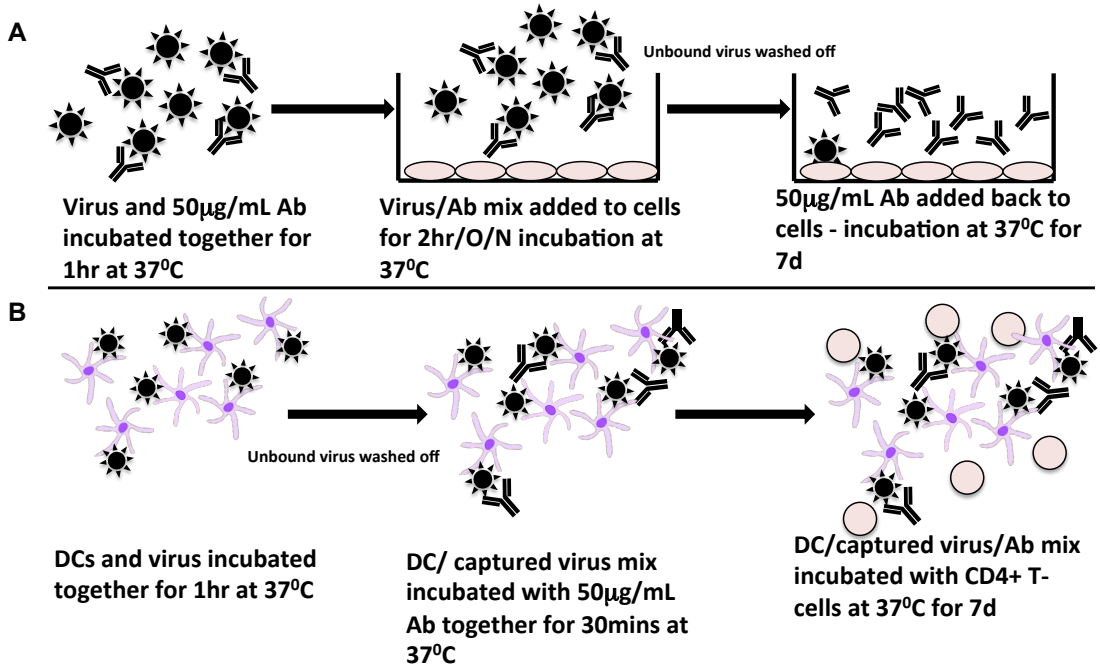


Figure 4.1 Inhibition assay setup to assess (A) direct infection of MDM, MDDC or PBMC and (B) *trans*-infection from DC-to-CD4+ T cells

Furthermore, the mucosal explant models investigate the ability of antibodies to prevent direct infection of penile glans, ectocervical and colorectal

tissue. After overnight infection, cells that have migrated out of the mucosal explants are collected and used in assay in a similar set up to the DC-T experiment, to investigate the ability of antibodies to prevent onward transmission from mucosal migratory cells to CD4+ T cells (Figure 4.2).

Supernatants were collected on day 7 (cellular and migratory models) or 21 (mucosal explant models) and analysed for p24 concentration (ELISA) as an indication of viral replication. Results were calculated by comparing p24 levels detected in the positive control well (tissue, plus virus-only) with the p24 levels in the experimental wells to give a percentage of infection value:

$$\%\ infection = \frac{p24\ concentration\ in\ test\ antibody\ well}{p24\ concentration\ in\ virus\text{-}only\ control\ well} \times 100$$

These results will help determine the ability of nAb and nnAb to inhibit HIV-1 infection at mucosal surfaces. This chapter forms the basis of a paper [438].

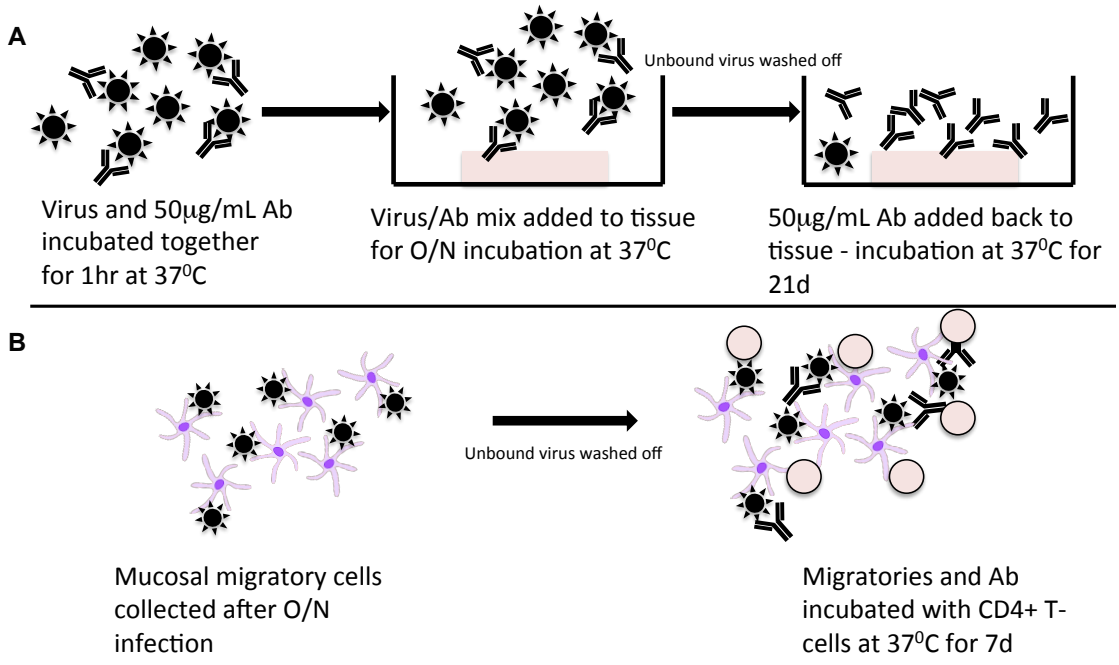


Figure 4.2 Inhibition assay setup in (A) mucosal tissue explants and (B) migratory cell-CD4+ T cells

The aims of this chapter are to:

1. Assess the activity of nAb and nnAb at inhibiting direct HIV-1 infection of PBMC and PBMC-derived macrophages and DCs.
2. Assess the ability of nAb and nnAb at inhibiting *trans*-infection of CD4+ T cells by DCs.
3. Assess the ability of nAb and nnAb to prevent direct infection of penile glans, ectocervical and colorectal tissue explants.
4. Assess the ability of nAb and nnAb at inhibiting onward infection of CD4+ T cells from infected migratory cells collected from penile glans, ectocervical and colorectal tissue explants.

4.2. Results

4.2.1. FcR Expression Profile of Macrophages, DCs and PBMC Used in the Inhibition Assays.

Macrophages and DCs play an important role in the early stages of HIV-1 transmission and the cellular inhibition models utilised in this chapter have been designed to mimic the early stages of infection. Macrophages and DCs were derived from PBMC (described in sections 2.4.4 and 2.4.5). Before using the cells in the inhibition assays, it was important to characterise the FcR expression phenotypes, as previously described in Chapter 3 for penile glans, ectocervical and colorectal tissue and whole blood. To this end, cells were stained using the FcR flow cytometry panel listed in Table 2.2 and acquired on a BD FortessaLSR flow cytometer before being analysed for the expression of FcR CD16, CD32, CD64 and CD89 on total viable cells.

High levels of expression of CD32, CD64 and CD89 were observed in all cell types, with good expression of CD16 also noted (Figure 4.3A). Boolean gating analysis to investigate the combinatorial nature of the FcR indicated that the majority of macrophages were triple positive for CD32, CD64 and CD89, whereas DCs and PBMC were more diverse in their FcR expression (Figure 4.3B). However, all cell types demonstrated high levels of FcR expression, with very few macrophages or DCs and only around 16% of the PBMC exhibiting a FcR-negative phenotype. This is in contrast to the large FcR-negative populations previously observed in mucosal tissues in Chapter 3.

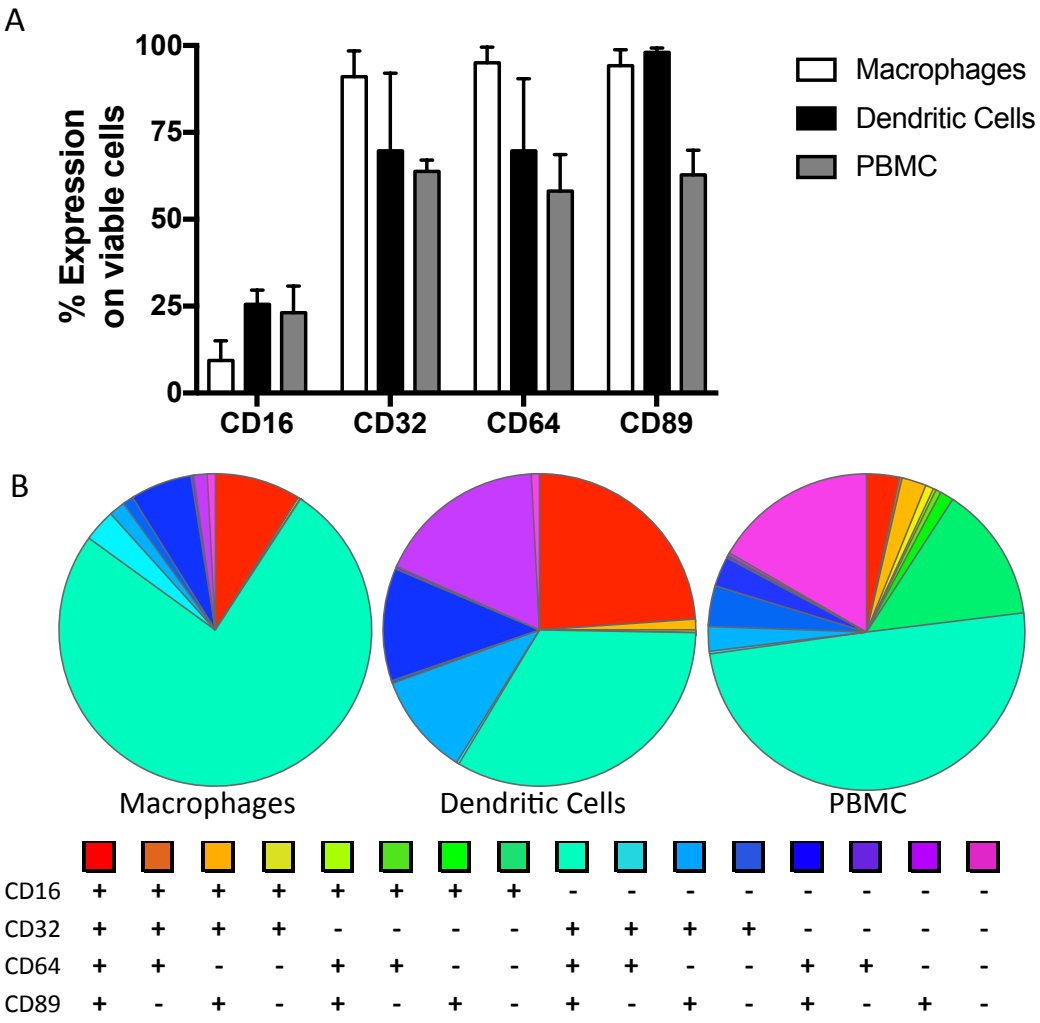


Figure 4.3 Fc receptor phenotyping of macrophages, DCs and PBMC used in the cellular inhibition assays.

(A) Flow cytometry analysis of the percentage expression of CD16, CD32, CD64 and CD89 FcR expression on total viable macrophages, DCs and PBMC used in the cellular inhibition assays (n=3). (B) Boolean gating analysis of the FcR expression on total viable cells to show the combinatorial variability of the Fc receptors.

4.2.2. Assessment of HIV-1 Specific Antibodies in Cellular Inhibition Assays.

The Center for HIV/AIDS Vaccine Immunology (CHAVI) consortium have isolated and characterised multiple HIV-1 specific tier-1 nAb and nnAb from participants of the RV144 and subsequent vaccine trials. For the purpose of this study, a large panel of non- or weakly-neutralising IgG and IgA antibodies from the CHAVI consortium (Table 4.1) were screened to assess their relative abilities to inhibit direct infection of macrophages and DCs, or to block the onward transmission from DC-to-CD4+ T cells. The backbone of some of the HIV-1 specific IgG1 antibodies included the S298A/E333A/K334A mutations in the IgG1 constant region, denoted by AAA, which improves FcR engagement and enhances effector function, in particular ADCC [439].

To ensure that antibody inhibitory activity was detected, a high antibody concentration of 50 μg/mL was used to set up all cellular inhibition assays with HIV-1_{BaL} and analysed using p24 ELISA. p24 levels in the positive control (virus plus cells, only) were compared to the p24 levels in the antibody samples to provide a percentage of infection score, with 100% denoting no reduction in HIV-1_{BaL} in the supernatant and 0% denoting a reduction in p24 to undetectable levels. Comparisons of these percentage scores to influenza-specific CH65 IgG1 or CH65 mIgA isotype control antibodies using one-way ANOVA, with Dunnett's correction for multiple comparisons enabled assessment of antibody inhibitory function in terms of statistical significance.

Direct infection of macrophages was significantly inhibited by 7B2 AAA (*P<0.05), CH20 AAA (****P<0.0001) and CH28 AAA (****P<0.0001) when compared to the IgG1 influenza-specific control, CH65 AAA. Interestingly, in

contrast to 7B2 AAA IgG, 7B2 mIgA2 and dIgA2 failed to show any ability to reduce infection in macrophages (Figure 4.5A). As the antigen binding epitopes of these antibodies are identical and only their FcR-binding abilities differ, this hints at a potential role for FcR-induced effector functions and signalling through FcγR as a mechanism of inhibition for these antibodies.

Although no statistically significant levels of inhibition were noted in the DC model, an average inhibition of 61.6% was determined for CH22 AAA IgG (Figure 4.4B & Table 4.2). No inhibition was detected for any of the antibodies assessed in the DC-to-CD4+ T cell *trans*-infection model (Figure 4.4C).

IgG antibodies F240, N10-U1 and M785-U1 demonstrated good levels of inhibition in both the macrophage and DC direct-infection models (Figure 4.4A & B). However, due to limited antibody availability, it was only possible to perform these experiments once, therefore preventing these results from reaching statistical significance. Intriguingly, these antibodies did not show any ability to inhibit *trans*-infection in the DC-to-CD4+ T cell model (Figure 4.4C). With fewer effector cells available in this model, a potential role for FcR binding and further downstream effector cell functions such as ADCC, ADCP or ADCVI in the inhibitory activity of these antibodies is possible

As none of the IgA antibodies demonstrated any ability to inhibit infection in these cellular models, they were not used in subsequent assays (Figure 4.5). Furthermore, as there were no IgG antibodies that displayed clear inhibitory activity across all three cellular models, results were instead used to inform the development of antibody cocktails designed to work synergistically with the aim of enhancing the inhibitory effect seen by nnAb. Although CH20 and CH28 performed the best in the macrophage model, these antibodies were

not used in future experiments due to limited antibody availability. Therefore, based on the combination of data obtained in these experiments and information from literature searches, IgG1 antibodies 7B2AAA, CH58, CH90AAA, CH22AAA, F240, N10-U1 and M785-U1 were selected for use in antibody cocktails. The gp41-specfic antibody 7B2AAA showed an ability to reduce HIV-1_{BaL} infection in macrophages by a mean value of 75.9% (Table 4.2), with additional studies demonstrating inhibitory activity for 7B2AAA in *in vitro* models and also ADCC activity [240]. CH22AAA, a gp120-V3-specific antibody demonstrated limited ability to inhibit HIV-1_{BaL} infection in DCs and had additionally been shown to have tier-1 neutralising abilities [440]. While CD4i C1-specific CH90AAA and gp120-V2-specific CH58 showed no inhibitory abilities in any of the cellular models, other studies have shown these antibodies to have good ADCC-inducing abilities and, for CH58, to have a tier-1 neutralising ability [441]. Furthermore, a previous study investigating the ability of HIV-1 specific antibodies to capture infectious virions showed an increased inhibitory effect was achieved when 7B2AAA and CH58 antibodies were combined [442]. As such, antibody cocktails containing 7B2AAA/CH58/CH90 and 7B2AAA/CH58/CH22, all with different epitope specificities, were assembled. Additionally, gp41-specific F240, N10-U1 and M785-U1, which showed good inhibitory activity in the macrophage and DC assays (Figure 4.4) and have also been shown by others to exhibit good ADCC-activity [443], were combined to assess for enhanced inhibitory ability.

Table 4.1 Antibodies for assessment in cellular inhibition assays.

Ab ID	Isotype	Epitope	Comments
7B2 AAA	G1/mA2/dA2	gp41 (Cluster I)	Non-neutralising
A32 CL4	G1	CD4i C1 (Cluster A)	Non-neutralising; Good ADCC
CH01 AAA	G1	gp120, V1V2/V2V3	Tier-1 neutralising
CH20 AAA	G1	CD4i C1; non-A32 blockable	Non-neutralising
CH22 AAA	G1	gp120 V3, linear	Tier-1 neutralising
CH23 AAA	G1	gp120 V3, linear	Tier-1 neutralising
CH27 AAA	G1/A2	Consensus C.Con gp140 Env	Non-neutralising
CH28 AAA	G1/A2	DU123/Con-S A2, VH3-66	Non-neutralising
CH29	G1/A2	CD4i C1, A32-blockable	Non-neutralising
CH38 AAA	G1/A2	CD4i C1, A32-blockable	Non-neutralising
CH54	G1	CD4i C1, A32-blockable	Non-neutralising
CH57	G1	CD4i C1, A32-blockable	Non-neutralising
CH58	G1	gp120, V2	Tier-1 neutralising
CH59	G1	gp120, V2	Tier-1 neutralising
CH65 AAA	G1/A2	HA, H1	Influenza-specific control
CH90 AAA	G1	CD4i C1, A32-blockable	Non-neutralising
CH115	A2	gp41	Non-neutralising
F240*	G1	gp41 (Cluster I)	Non-neutralising
HG116	G1	gp120	Non-neutralising
HG118	G1	gp120	Non-neutralising
HG120	G1	gp120, V2	Non-neutralising
HG129	A2	C1 linear peptide	Non-neutralising
HG130	A2	Consensus S gp140 Env.	Non-neutralising
M785-U1*	G1	gp41	Non-neutralising
N10-U1*	G1	gp41	Non-neutralising

Antibodies isolated from HIV-1 vaccine participants or HIV-1 positive individuals by the CHAVI consortium or kindly provided by G. K. Lewis, University of Maryland, USA (*). AAA denotes IgG1 antibodies containing the S298A/E333A/K334A mutations in the IgG1 constant region, which have been shown to improve ADCC activity.

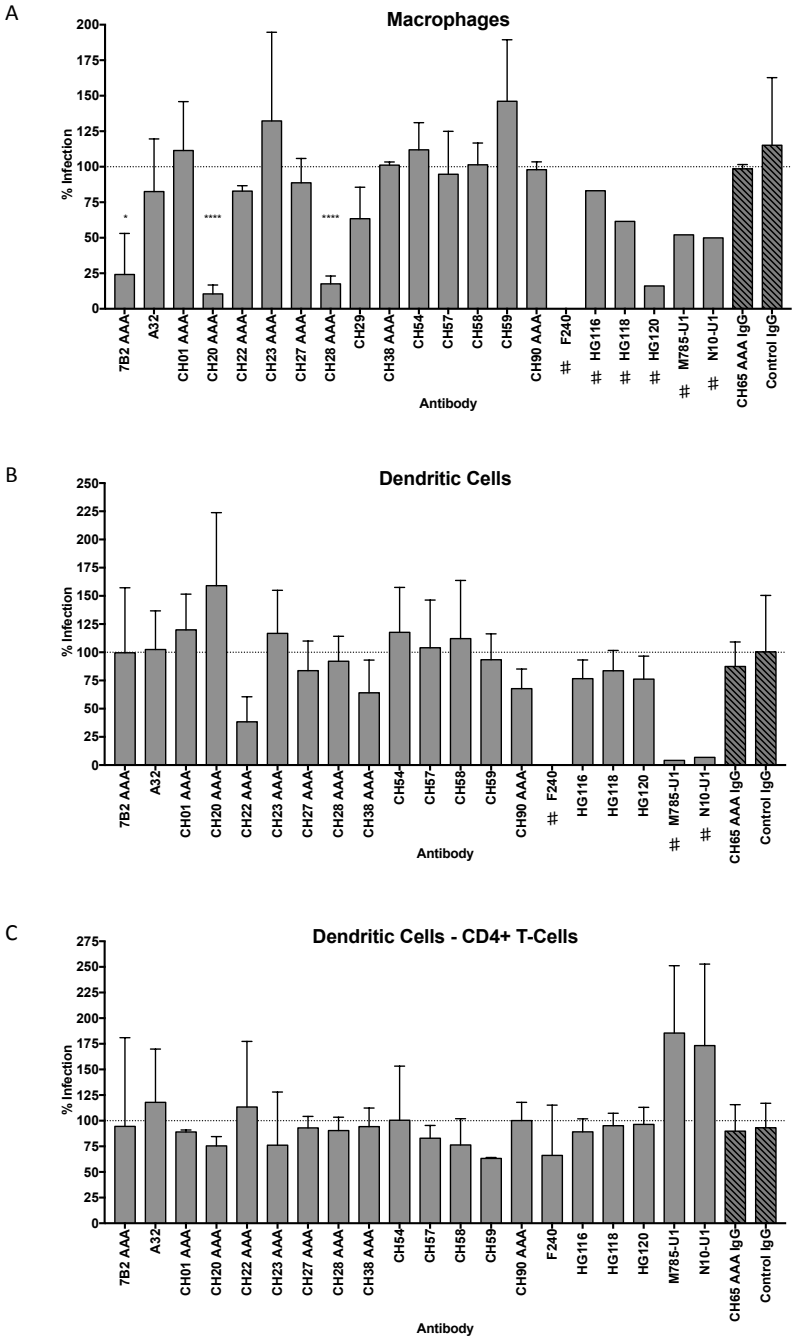


Figure 4.4 Assessment of IgG antibodies in cellular models

The ability of IgG antibodies (50 μg/mL) to reduce levels of HIV-1_{BaL} infection in (A) Macrophages (n=3; except ⌗, where n=1), (B) DCs (n=3; except ⌗, where n=1) or (C) DC-to-CD4+ T cell models (n=3). Graphs show percentage infection in comparison to the infected, media-only control. Statistical analysis performed using one-way ANOVA with Dunnett's multiple comparison correction comparing individual antibodies to the CH65 IgG1 influenza-specific control monoclonal antibody. [*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001]

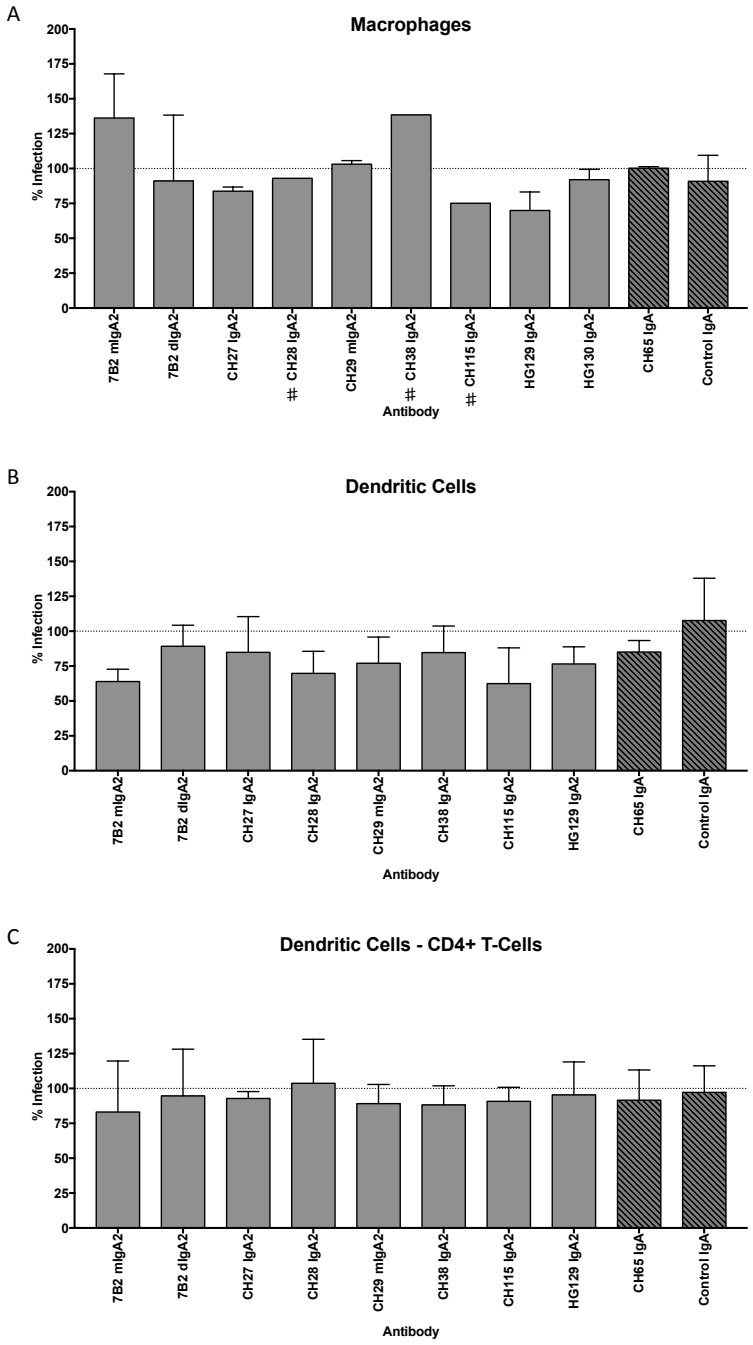
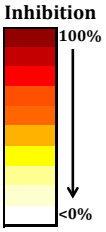


Figure 4.5 Assessment of IgA antibodies in cellular models

The ability of IgA antibodies (50 μg/mL) to reduce levels of HIV-1_{BaL} infection in (A) Macrophages (n=3; except #, where n=1), (B) DCs (n=3) or (C) DC-to-CD4+ T cell models (n=3). Graphs show percentage infection in comparison to the infected, media only control. Statistical analysis performed using one-way ANOVA with Dunnett's multiple comparison correction comparing individual antibodies to the CH65 mIgA2 influenza-specific control monoclonal antibody.

Table 4.2 Summary of percentage inhibition to HIV-1_{BaL} detected across the cellular inhibition assays

Antibody	Antibody Specificity	Inhibition Assay					
		Macrophages		*Dendritic Cells*		*DC-T Cells*	
		Mean	SD	Mean	SD	Mean	SD
7B2 AAA	gp41 (Cluster I)	75.9	±29.0	0.4	±57.6	5.6	±86.5
A32	CD4i C1 (Cluster A)	17.5	±37.1	-2.4	±34.3	-18.0	±52.0
CH01 AAA	gp120, V1-V2/V2-V3	-11.5	±34.4	-19.9	±31.6	10.9	±1.9
CH20 AAA	CD4i C1; non-A32 blockable	89.5	±6.3	-59.1	±64.7	24.6	±9.1
CH22 AAA	gp120 V3, linear	17.1	±3.7	61.6	±22.2	-13.4	±64.0
CH23 AAA	gp120 V3, linear	-32.3	±62.4	-16.7	±38.2	23.9	±51.9
CH27 AAA	Consensus C.Con gp140 Env	11.3	±17.1	16.3	±26.2	7.0	±11.2
CH28 AAA	DU123/Con-S A2, VH3-66	82.5	±5.6	7.9	±22.2	9.6	±13.1
CH29	CD4i C1, A32-blockable	36.5	±22.1	ND	ND	ND	ND
CH38 AAA	CD4i C1, A32-blockable	-1.1	±2.3	36.0	±29.1	5.7	±18.1
CH54	CD4i C1, A32-blockable	-11.9	±19.2	-17.7	±39.9	-0.4	±52.8
CH57	CD4i C1, A32-blockable	5.3	±30.3	-4.0	±42.3	17.1	±12.4
CH58	gp120, V2	-1.4	±15.4	-12.1	±51.6	23.6	±25.6
CH59	gp120, V2	-46.1	±43.3	6.6	±23.0	36.7	±0.9
CH90 AAA	CD4i C1, A32-blockable	2.1	±5.6	32.2	±17.3	-0.1	±17.8
F240	gp41 (Cluster I)	100.0	N/A	100.0	N/A	33.9	±49.1
HG116 AAA	gp120	16.9	N/A	23.4	±16.6	10.9	±12.7
HG118 AAA	gp120	38.4	N/A	16.3	±17.9	4.9	±12.1
HG120 AAA	gp120, V2	83.9	N/A	23.8	±20.3	3.6	±16.6
M785-U1	gp41	47.9	N/A	95.8	N/A	-85.5	±65.7
N10-U1	gp41	50.1	N/A	93.1	N/A	-73.2	±79.5
CH65 AAA IgG1	Flu Haemagglutinin	1.5	±3.0	12.6	±21.7	10.2	±25.9
Control IgG	Polyclonal IgG	-15.1	±47.6	-0.3	±52.6	6.9	±23.9
7B2 mIgA2	gp41 (Cluster I)	-36.2	±31.6	36.0	±8.8	16.9	±36.6
7B2 dIgA2	gp41 (Cluster I)	8.9	±47.2	10.7	±15.0	5.3	±33.5
CH27 IgA2	Consensus C.Con gp140 Env	16.2	±2.9	15.1	±25.6	7.1	±4.9
CH28 IgA2	DU123/Con-S A2, VH3-66	7.0	N/A	30.2	±15.9	-3.7	±31.5
CH29 mIgA2	CD4i C1, A32-blockable	-3.1	±2.6	23.0	±18.8	10.9	±13.8
CH38 IgA2	CD4i C1, A32-blockable	-38.5	N/A	15.3	±19.0	11.8	±13.7
CH115 IgA2	gp41	24.9	N/A	37.6	±25.6	9.2	±10.0
HG129 IgA2	C1 linear peptide	30.0	±13.2	23.5	±12.3	4.6	±23.7
HG130 IgA2	Consensus S gp140 Env.	8.0	±7.4	ND	ND	ND	ND
CH65 mIgA2	Flu Haemagglutinin	-0.2	±1.1	14.9	±8.2	8.4	±21.6
Control IgA	Polyclonal IgA	9.1	±18.6	-7.7	±36.5	2.8	±19.0



Antibodies assessed at 50 μg/mL. Mean values are shown with SD. Highlighted cells <2SD. ND = Not done. N/A = Not applicable due to n=1.

4.2.3. NAb and nnAb Panel Design

Leading on from the development of nnAb cocktails, the next stage was to investigate and compare and contrast the relative abilities of nAb in preventing HIV-1_{BaL} infection with those of the nnAb cocktails. To this end, a panel of single, monoclonal nAb and nnAb was developed, comprising of antibodies with specificities to known major HIV-1 neutralising epitopes including CD4bs, gp120 glycans and MPER. Antibody isotypes IgG1, mIgA1, mIgA2 and dIgA2 were tested and, unless specified, antibodies are of the IgG1 isotype in all subsequent results. For completeness, polyclonal HIV-IG antibodies to clade B and clade C viruses were also compared to determine their relative impact on inhibiting HIV-1_{BaL} infection (Table 4.3). A32, a known efficient ADCC-inducing antibody [240, 444] was also included as a comparative control, as were influenza-specific CH65 IgG and IgA isotypes. All antibodies within the panel were screened on the cellular inhibition assays and further tested for inhibitory activity on mucosal explants and mucosal migratory-to-CD4+ T cell models.

Table 4.3 Panel of nAb and nnAb used in the cellular and mucosal explant models.

Antibody	Antibody Specificity
VRC01 IgG ***CH31IgG*** ***CH31 mIgA1*** ***CH31 mIgA2*** ***CH31 dIgA2*** ***b12 IgG***	CD4-binding site
2F5 IgG ***2F5 dIgA*** ***4E10 IgG*** *4B3 IgG*	Membrane-Proximal External Region (MPER)/gp41
2G12 IgG ***PG9 IgG*** ***PG16 IgG***	Glycan
7B2/CH58/CH90 *7B2/CH58/CH22* *F240/M785-U1/N10-U1*	Various (IgG)
HIV-IG B *HIV-IG C*	Polyclonal
A32 IgG	CD4i Cluster 1
CH65 IgG *CH65 IgA*	Flu Haemagglutinin

Antibodies were used in assay at 50 μg/mL for the single antibodies or 25 μg/mL (each) for the antibody cocktails. Bold = Neutralising Antibodies.

4.2.4. Antibody Neutralisation in PBMC and TZM-bl Cells

Initial inhibitory function of the antibodies was assessed at 50 μg/mL to ensure the antibodies were at a high enough concentration to detect inhibitory activity. Once the nAb and nnAb panel had been designed (Table 4.3), it was important to further define the neutralising abilities of these antibodies through titration on TZM-bl indicator cells and PBMC. The use of two different cell types enabled an assessment of the neutralisation capacities of the antibodies in an environment void of FcR and effector cells (TZM-bl) i.e. classical neutralisation, with an environment where FcR and effector cells were present (PBMC).

The CD4bs antibodies demonstrated good neutralisation ability in TZM-bl cells, with IC90 values of <50 μg/mL determined for all antibodies tested. Indeed, VRC01 IgG, CH31 IgG and b12 IgG achieved IC90 values of 0.087 μg/mL, 6.125 μg/mL and 0.070 μg/mL, respectively (Figure 4.6A & Table 4.4). The neutralisation data for CD4bs antibodies obtained from PBMC also showed good ability to neutralise HIV-1_{BaL}, even at low concentrations (Figure 4.7A). Interestingly, the CH31 IgA isotype antibodies were not as efficient at neutralising virus in either cell type when compared to the CH31 IgG isotype (Figures 4.6 and 4.7).

The MPER/gp41-specific antibodies did not demonstrate any ability to neutralise HIV-1_{BaL} in the TZM-bl cells (Figure 4.6B). However, when tested on PBMC, 2F5 IgG and 4E10 IgG demonstrated good neutralisation ability, with observed IC90 values of 25.204 μg/mL and 24.416 μg/mL, respectively (Figure 4.7B). In contrast, 4B3 IgG and 2F5 dIgA did not show any ability to neutralise in PBMC.

Glycan-specific antibodies PG9 IgG and PG16 IgG demonstrated good neutralisation of HIV-1_{BaL} in TZM-bl cells, with IC90 values of 0.036 μg/mL and 0.243 μg/mL, respectively, whereas no neutralisation was observed for 2G12 IgG (Figure 4.6C and Table 4.4). When tested on PBMC, all of the assayed glycan-specific antibodies demonstrated an ability to neutralise HIV-1_{BaL} (Figure 4.7C). PG9 IgG and PG16 IgG showed greater neutralisation abilities when compared to 2G12 IgG, however, IC90 values of 0.019 μg/mL, 9.274 μg/mL and 24.735 μg/mL, respectively, were determined for these antibodies in PBMC (Table 4.4).

No neutralisation was observed for any of the nnAb cocktails tested on TZM-bl cells (Figure 4.6D). However, when assayed on PBMC, IC80 values of 11.324 μg/mL and 18.775 μg/mL were observed for 7B2/CH58/CH90 and 7B2/CH58/CH22, respectively (Table 4.4). Similarly, ADCC-control, A32, demonstrated no inhibitory activity in TZM-bl cells (Figure 4.6F), but an IC50 value of 28.256 μg/mL was determined in PBMC (Figure 4.7F and Table 4.4).

Polyclonal HIV-IG antibodies were not able to neutralise HIV-1_{BaL} in TZM-bl cells (Figure 4.6E). However, as was observed for the nnAb cocktails, 7B2/CH58/CH90 and 7B2/CH58/CH22, both HIV-IG B and C demonstrated an ability to neutralise HIV-1_{BaL} in PBMC (Figure 4.7E and Table 4.4).

The dichotomy in neutralisation observed between TZM-bl cells and PBMC, in particular for the nnAb cocktails and HIV-IG antibodies, but also within the CD4bs-, MPER- and glycan-specific antibodies, could indicate the use of FcR and downstream effector functions in the PBMC assay.

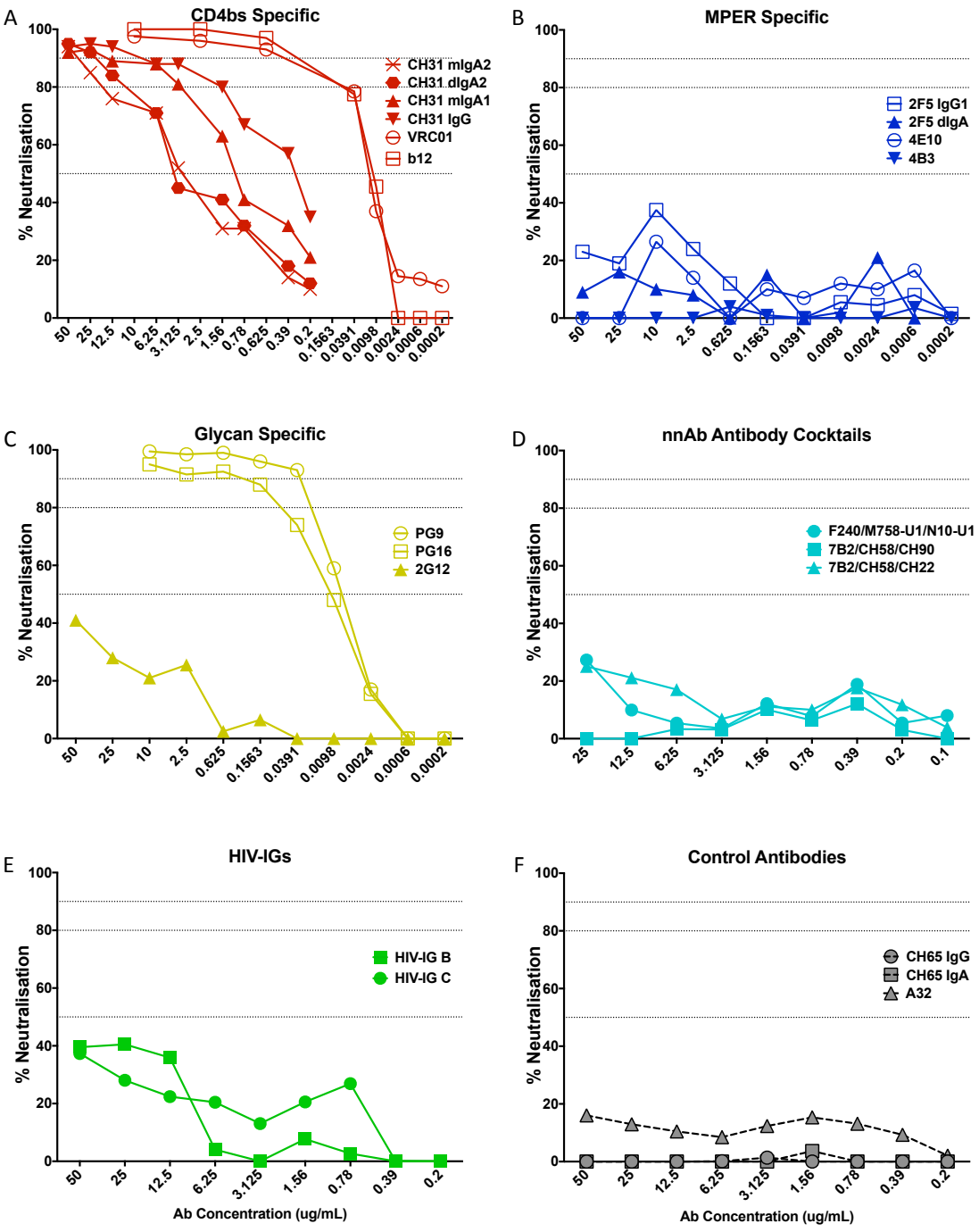


Figure 4.6 Neutralisation data in TZM-bl cells

Percentage neutralisation curves for HIV-1 specific antibodies titrated from a starting concentration of 50 μg/mL for single antibodies (10 μg/mL for b12, VRC01, PG9 & PG16,) or 25 μg/mL (each) for the antibody cocktails in TZM-bl indicator cells (n=3). Mean values shown.

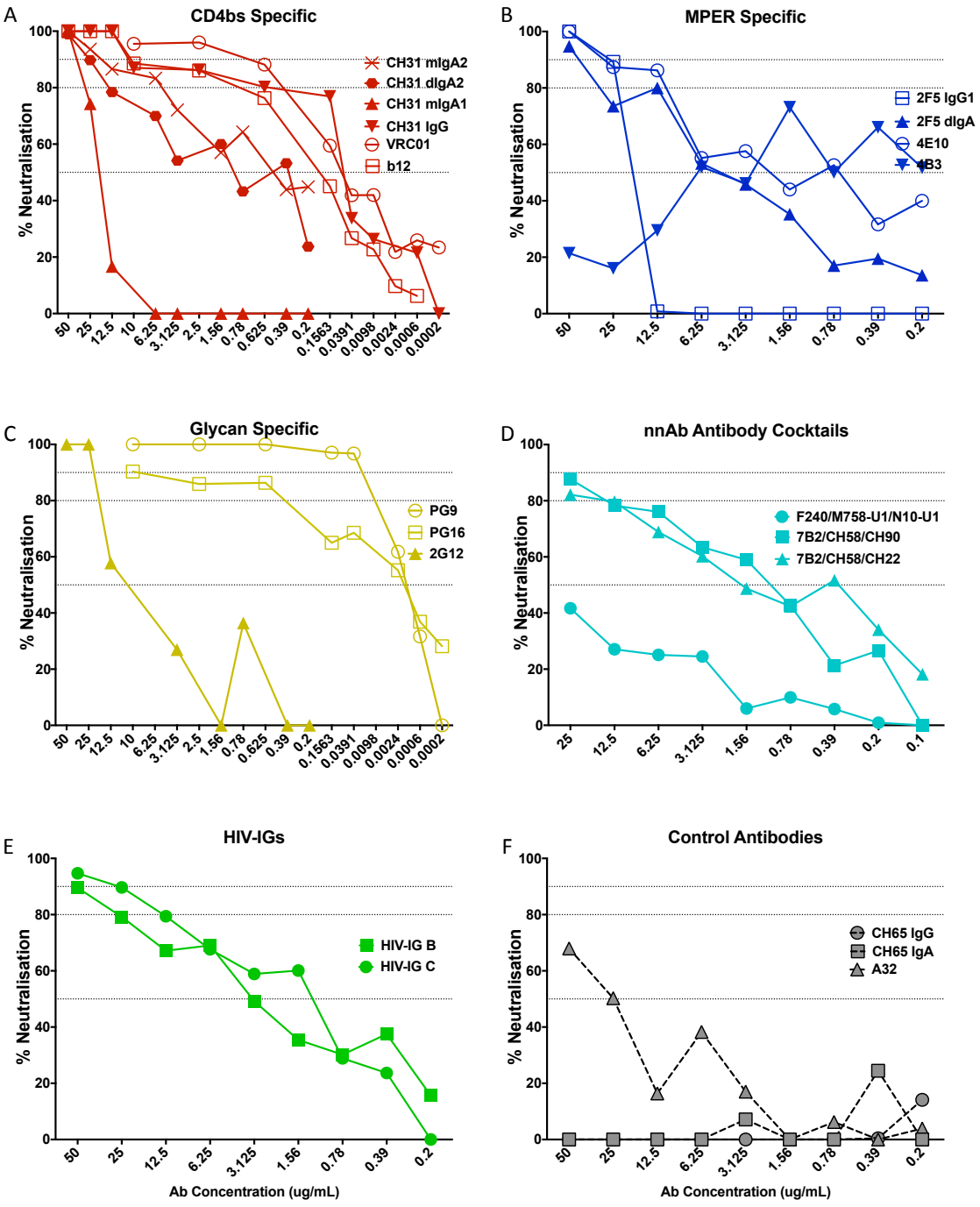


Figure 4.7 Neutralisation data in PBMC

Percentage neutralisation curves for HIV-1 specific antibodies titrated from a starting concentration of 50 μg/mL for single antibodies (10 μg/mL for b12, VRC01, PG9 & PG16,) or 25 μg/mL (each) for the antibody cocktails in PBMC (n=3). Mean values shown.

Table 4.4 Summary of neutralisation data in TZM-bl cells and PBMC

Antibody	Antibody Specificity	IC50		IC80		IC90	
		TZM-bl	*PBMC*	*TZM-bl*	*PBMC*	*TZM-bl*	*PBMC*
VRC01 IgG	CD4-binding site	0.015	0.062	0.042	0.465	0.087	1.253
CH31IgG		0.322	0.060	1.621	0.444	6.125	1.643
CH31 mIgA1		1.009	19.091	3.162	27.024	9.432	32.511
CH31 mIgA2		2.867	0.439	14.052	6.133	35.205	15.528
CH31 dIgA2		2.671	1.076	11.506	13.287	23.082	27.912
b12 IgG		0.013	0.167	0.036	1.243	0.070	3.638
2F5 IgG	Membrane-Proximal External Region (MPER)/gp41	X	20.219	X	23.236	X	25.204
2F5 dIgA		X	X	X	X	X	X
4E10 IgG		X	2.253	X	13.963	X	24.416
4B3 IgG		X	X	X	X	X	X
2G12 IgG	Glycan	X	8.731	X	18.289	X	24.735
PG9 IgG		0.007	0.001	0.019	0.007	0.036	0.019
PG16 IgG		0.011	0.002	0.056	0.536	0.243	9.274
7B2/CH58/CH90	Various (IgG)	X	1.159	X	11.324	X	X
7B2/CH58/CH22		X	1.018	X	18.775	X	X
F240/M785-U1/N10-U1		X	X	X	X	X	X
HIV-IG B	Polyclonal	X	2.984	X	24.393	X	X
HIV-IG C		X	1.573	X	12.045	X	31.537
A32 IgG	CD4i Cluster 1	X	28.256	X	X	X	X
CH65 IgG	Flu Haemagglutinin	X	X	X	X	X	X
CH65 IgA		X	X	X	X	X	X

IC50, IC80 and IC90 values (μg/mL) determined from neutralisation data of HIV-1 specific antibodies in TZM-bl cells (n=3) or PBMC (n=3). X = no neutralisation seen at 25 μg/mL (antibody cocktails) or 50 μg/mL (single antibodies).

4.2.5. Inhibition in Cellular Models

Although the previous TZM-bl and PBMC titration data informed on the relative neutralisation abilities of the different antibodies, to ensure inhibition was seen in the subsequent inhibition models, a high concentration of 50 μg/mL was used. Initially, the inhibitory activities of single antibodies and antibody cocktails were investigated in the same macrophage and DC models used in section 4.2.2, with the addition of a PBMC model to determine antibody inhibitory activity in a more complete cellular setting.

CD4bs antibodies were capable of significantly inhibiting direct infection of macrophages, with IgG and mIgA1 isotypes demonstrating a significance of ****P<0.0001 and IgA2 isotypes demonstrating a significance of *P<0.05 (Figure 4.8A). Direct infection of DCs was significantly inhibited by VRC01, CH31, b12 IgG and CH31 mIgA1 (**P<0.01), whereas no inhibition was observed for CH31 mIgA2 or dIgA2 (Figure 4.8B). *Trans*-infection from DC-to-CD4+ T cells was significantly inhibited by VRC01 and b12 IgG antibodies and CH31 mIgA2 and dIgA2 antibodies (**P<0.01) (Figure 4.8C). Although CH31 IgG and mIgA1 did not reach statistically significant levels of inhibition, they were capable of reducing infection by 66.3% and 52.8%, respectively (Table 4.5). All CD4bs antibodies were capable of high levels of inhibition in PBMC, with VRC01 IgG, CH31 IgG, mIgA1, mIgA2 and dIgA2 and b12 IgG demonstrating between 98.9-100% inhibition of HIV-1_{BaL} (Figure 4.8D and Table 4.5.).

MPER/gp41-specific antibodies, 2F5, 4E10 and 4B3 IgG all demonstrated significant levels of inhibition of direct infection of macrophages (****P<0.0001), while 2F5 dIgA was not able to inhibit in this model (Figure 4.8A and Table 4.5). Similarly, significant levels of inhibition were observed for 2F5 IgG and 4E10 IgG

in the direct DC infection assay (**P<0.01) (Figure 4.8B). While inhibition levels did not reach significance for 4B3 IgG in this assay, there was a mean inhibition of 73.9% (Table 4.5). As in the macrophage inhibition assay, no reduction of infection was observed for 2F5 dIgA in the DC assay (Figure 4.8B). MPER/gp41-specific antibodies 2F5 and 4E10 IgG showed good abilities to inhibit *trans*-infection of CD4+ T cells by DCs (**P<0.01). Although 2F5 dIgA and 4B3 IgG did not achieve significant levels of inhibition, they were able to reduce infection by 71.9% and 47.9%, respectively (Table 4.5). In PBMC, 2F5 and 4E10 IgG inhibited 100% of HIV-1_{BaL} infection, while 4B3 IgG was not able to significantly inhibit infection (Figure 4.8D). Due to limited antibody stock, it was not possible to assess the inhibitory ability of 2F5 dIgA in this assay model.

Glycan-specific IgG antibodies PG9, PG16 and 2G12 demonstrated high levels of inhibition in all cellular models (Figure 4.8). Direct infection of macrophages and DCs and *trans*-infection of CD4+ T cells was inhibited by 98.1-100% by these antibodies (Table 4.5). In the PBMC assay, 100% inhibition was observed by PG9 and 2G12, while PG16 reduced infection by 75% compared to the isotype control (Figure 4.8D).

The nnAb cocktails demonstrated significant levels of inhibition (****P<0.0001) in the macrophage assay, inhibiting infection by a mean of 90.7% (7B2/CH58/CH90), 95.8% (7B2/CH58/CH22) and 97.2% (F240/M785-U1/N10-U1) (Figure 4.8A and Table 4.5). In the DC model, nnAb cocktails 7B2/CH58/CH22 (*P<0.05) and F240/M785-U1/N10-U1 (**P<0.01) demonstrated significant levels of inhibition when compared to the CH65 IgG isotype control (Figure 4.8B). Although not significant, a mean inhibition of 56.5% was determined for 7B2/CH58/CH90 in DCs (Table 4.5). No significant

levels of inhibition were noted in the *trans*-infection DC-to-CD4+ T cell model (Figure 4.8C). In the PBMC model, 7B2/CH58/CH90 demonstrated significant levels of inhibition (*P<0.05) and, although the antibody cocktails 7B2/CH58/CH22 and F240/M785-U1/N10-U1 did not reach significance, they still demonstrated an ability to reduce HIV-1_{BaL} infection by 82.2% and 41.7%, respectively (Figure 4.8D and Table 4.5).

The HIV-IG B and HIV-IG C antibodies inhibited 100% of HIV-1_{BaL} infection in macrophages and DCs (Figure 4.8A and B). However, due to limited antibody availability, it was only possible to perform these experiments n=1, therefore the results did not reached statistical significance. In the *trans*-infection DC-to-CD4+ T cell model, HIV-IG B was not able to inhibit infection, whereas HIV-IG C significantly inhibited infection (**P<0.01) by an average of 89.5% (Figure 4.8C and Table 4.5). In PBMC, good levels of inhibition were observed for both HIV-IG antibodies, reducing levels of infection by 91.0% and 94.7%, respectively (Table 4.5).

ADCC-control antibody, A32, exhibited no inhibitory activity in the macrophage, DC or DC-to-CD4+ T cell models, but a mean neutralisation value of 83.5% was observed in PBMC (Table 4.5).

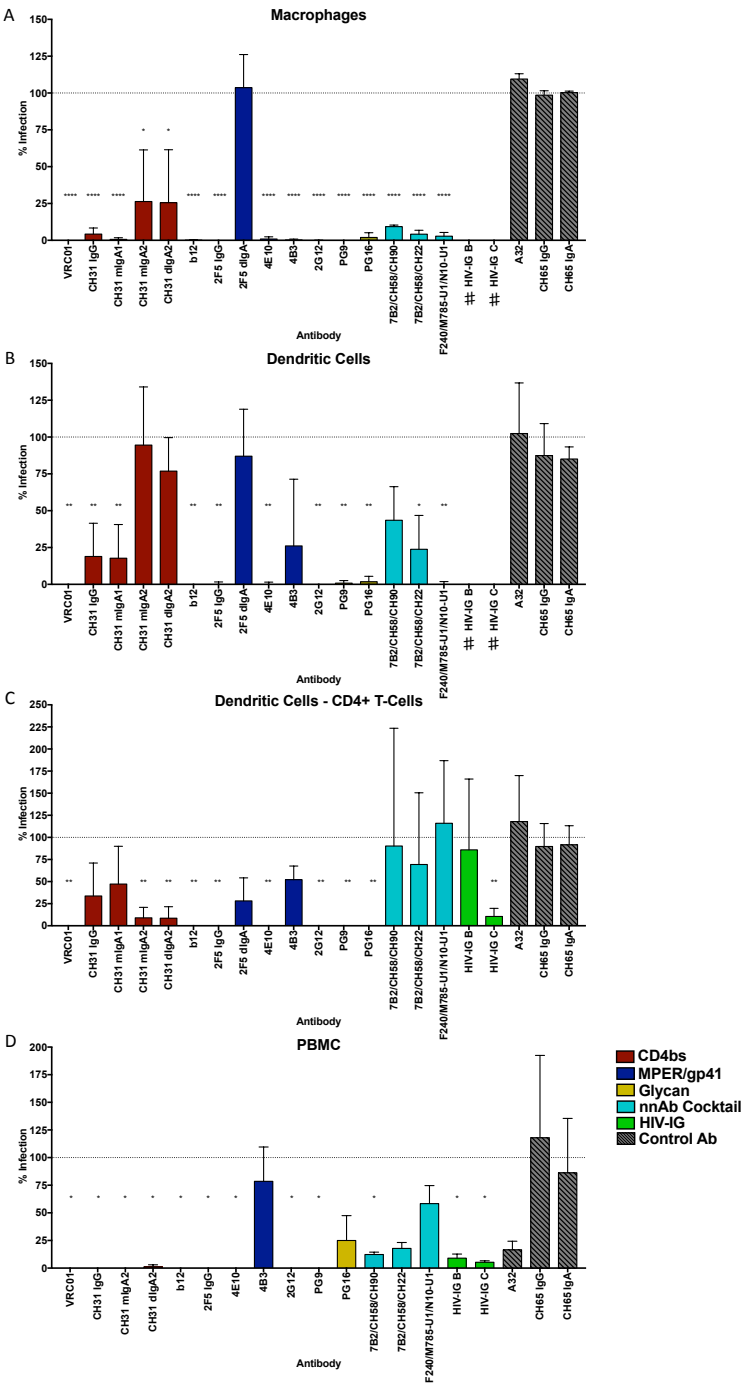


Figure 4.8 Inhibition of single antibodies and antibody cocktails in cellular inhibition assays

Inhibition of HIV-1_{BaL} by antibody panels in the direct infection of (A) macrophages (n=3) and (B) DCs (n=3), (C) *trans*-infection of CD4+ T cells from DCs (n=3) and (D) PBMC (n=3). Data is presented as percentage infection when compared to the HIV-1_{BaL}-positive control. One-way ANOVA with Dunnett's multiple comparison test followed by an unpaired t test was used to compare the antibodies with the CH65 isotype controls. [*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001]

4.2.6. Inhibition in Mucosal Tissue Explant Models

As the majority of HIV-1 infections are transmitted via mucosal surfaces, it was important to ascertain the ability of nAb and nnAb to inhibit direct infection of mucosal tissue. To this end, experiments were set up as described in Figure 4.2A.

All CD4bs antibodies tested were capable of significantly reducing the levels of infection detected in penile glans tissue when compared to the CH65 isotype controls (Figure 4.9A and Table 4.5). Within ectocervical tissue, VRC01, CH31 IgG and b12 IgG showed significant inhibitory abilities when compared to the isotype control (P**<0.01)(Figure 4.9B). Although the inhibition induced by CH31 mIgA2 and dIgA2 did not reach significance, levels of infection were reduced by 60.7% and 62.3%, respectively. Within colorectal tissue, there was a greater deviation observed within the CH65 isotype control meaning that, for the CD4bs antibodies, only CH31 mIgA2 reached significant levels of inhibition (P*<0.05) (Figure 4.9C). However, when compared to the penile glans and ectocervical tissue, similar percentages of inhibition were noted for VRC01 (86.0%), CH31 IgG (76.1%) and CH31 dIgA2 (69.6%) (Table 4.5). b12 IgG was also shown to reduce levels of infection by an average of 47.6%, although there was a high variability within the assay for this antibody.

MPER/gp41-specific antibodies were comparatively less efficient at inhibiting infection when compared to the CD4bs antibodies. In penile glans and ectocervical tissue, only 2F5 IgG demonstrated any significant ability to reduce infection (****P<0.0001 and **P<0.01, respectively) (Figure 4.9A and B). Due to the high variability in the CH65 isotype control antibody observed in colorectal tissue, none of the MPER/gp41-specific antibodies significantly inhibited

infection in this tissue (Figure 4.9C). However, it should be noted that there were good levels of inhibition seen throughout the MPER/gp41-specific panel, with an average reduction of infection of 94.0% 2F5 IgG, 91.3% 2F5 dIgA, 91.7% 4E10 IgG and 51.4% 4B3 IgG (Table 4.5).

Glycan-specific antibody, PG9, demonstrated good inhibition in penile glans and ectocervical tissue (***P<0.001) (Figure 4.9A and B), although this inhibitory ability was not observed in colorectal tissue (Figure 4.9C). PG16 did not significantly inhibit infection in any of the tissue models (Figure 4.9). Infection was reduced by 94.3% by 2G12 in colorectal tissue (Table 4.5), whereas this antibody was capable of reducing infection by 41.8% in penile glans tissue and 71.0% in ectocervical tissue.

The nnAb cocktails were not able to significantly reduce infection within the penile glans or ectocervical tissue models (Figure 4.9A and B). Interestingly, in colorectal tissue, the antibody cocktail F240/N10-U1/M785-U1 showed the ability to reduce infection by 89.7% (Figure 4.9C and Table 4.5), potentially due to higher numbers of CD14+ monocytic cells found in this tissue, as described in Chapter 3 (Figure 3.7).

Both HIV-IG antibodies demonstrated some ability to inhibit HIV-1_{BaL} infection in all of the tissue models (Figure 4.9). In penile glans tissue, similar levels of inhibition were observed for both HIV-IG antibodies, with HIV-IG B reducing infection by 62.5% and HIV-IG C by 59.2% (Table 4.5). Although not statistically significant, a similar pattern of inhibition was noted in ectocervical tissue, with HIV-IG B reducing infection by 52.1% and HIV-IG C by 65.1% (Table 4.5). A greater inhibitory effect was observed in colorectal tissue, with HIV-IG B significantly inhibiting HIV-1_{BaL} infection when compared to the isotype control

(*P<0.05) by 96.7%. Although HIV-IG C did not reach statistical significance in this model, a reduction of infection of 76.1% was determined (Table 4.5).

ADCC-control antibody, A32, exhibited limited inhibitory activity in the tissue explant models, where mean inhibition values of 42.3%, 57.7% and 77.5% were observed in penile glans, ectocervical and colorectal tissue, respectively (Table 4.5).

Overall, the CD4bs antibodies demonstrated the widest and most complete ability to lower infection across the three tissue models investigated.

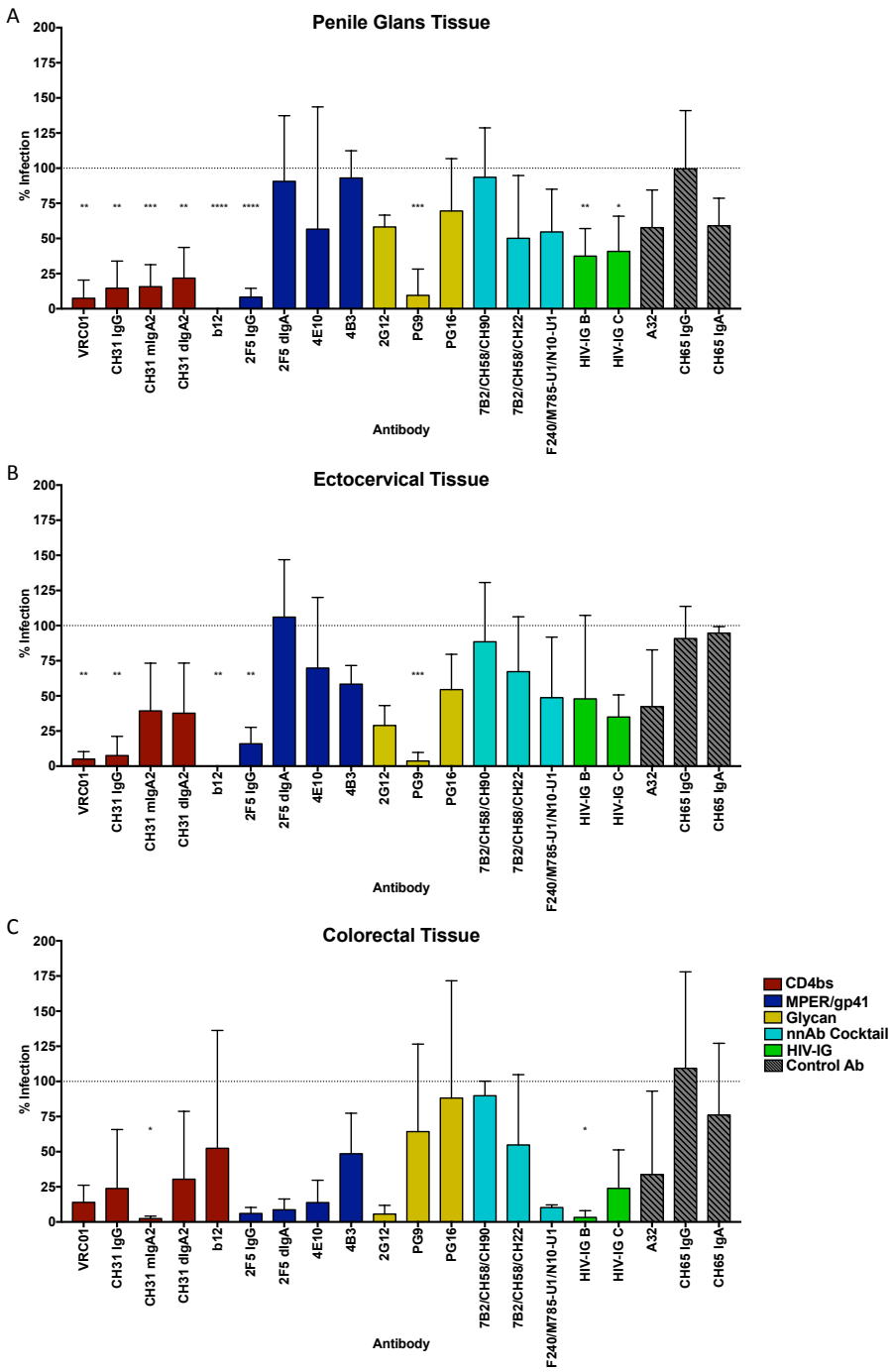


Figure 4.9 Inhibition by single antibodies and antibody cocktails in mucosal tissue explants.

Inhibition of HIV-1_{BaL} by antibody panels in the direct infection of (A) penile glans (n=3), (B) ectocervical (n=2 HIV-IG B & C; n=3, remaining Ab) and (C) colorectal tissue (n=3). Data is presented as percentage infection when compared to the HIV-1_{BaL}-positive control. One-way ANOVA with Dunnett's multiple comparison test followed by an unpaired t test was used to compare the antibodies with the CH65 isotype controls. [*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001]

4.2.7. Inhibition Assay Designed to Mimic Onward Transmission from Mucosal Tissue to CD4+ Target Cells.

During early HIV-1 transmission events at mucosal surfaces, cells migrate away from the site of primary infection and infect CD4+ target cells. To determine the ability of HIV-1 antibodies at preventing this onward transmission, cells that had migrated out of tissue explants during the overnight infection were collected and set up in culture with CD4+ target cells, as described in Figure 4.2B.

Across all tissue migratory cell models, both IgG and IgA isotypes of CD4bs-specific antibodies were very efficient at blocking the onward transmission of HIV-1 to the CD4+ target cells, with percentage inhibition levels ranging between 89.4-100% (Figure 4.10 and Table 4.5).

The MPER/gp41-specific antibodies were less efficient at inhibiting infection, with only 2F5 IgG demonstrating any significant ability to block onward infection of HIV-1_{BaL} from penile glans (82.3%; *P<0.05), ectocervical (86.1%; ***P<0.001) and colorectal (100%; ***P<0.001) migratory cells (Figure 4.10 and Table 4.5).

Glycan-specific antibodies showed a more diverse ability to inhibit onward infection. A significant reduction of 86.4% [***P<0.001] was observed in ectocervical migratory cells (Figure 4.10B). Although not statistically significant, 2G12 demonstrated an ability to reduce onward infection by mean percentages of 61.2%, and 73.7% in penile glans and colorectal migratory cells, respectively (Table 4.5). PG9 did not show any ability to block onward transmission of infection from penile glans (Figure 4.10A) or colorectal (Figure 4.10C) migratory cells. However, a good level of inhibition was observed in the ectocervical

migratory cell model (95.7%; ****P<0.0001) (Figure 4.10B and Table 4.5). PG16 was not able to block HIV-1_{BaL} transmission from penile glans or colorectal migratory cells. While not statistically significant, a mean inhibition percentage reduction of 54.2% was determined in the ectocervical cell model (Table 4.5).

Interestingly, similar to the results observed in DC-to-CD4+ T cell model, the nnAb cocktails showed no inhibitory ability in blocking onward infection in any of the migratory tissue cell models (Figure 4.10).

HIV-IG and ADCC-control, A32, antibodies were not able to significantly inhibit the onward transmission of HIV-1_{BaL} in any of the migratory cell models (Figure 4.10).

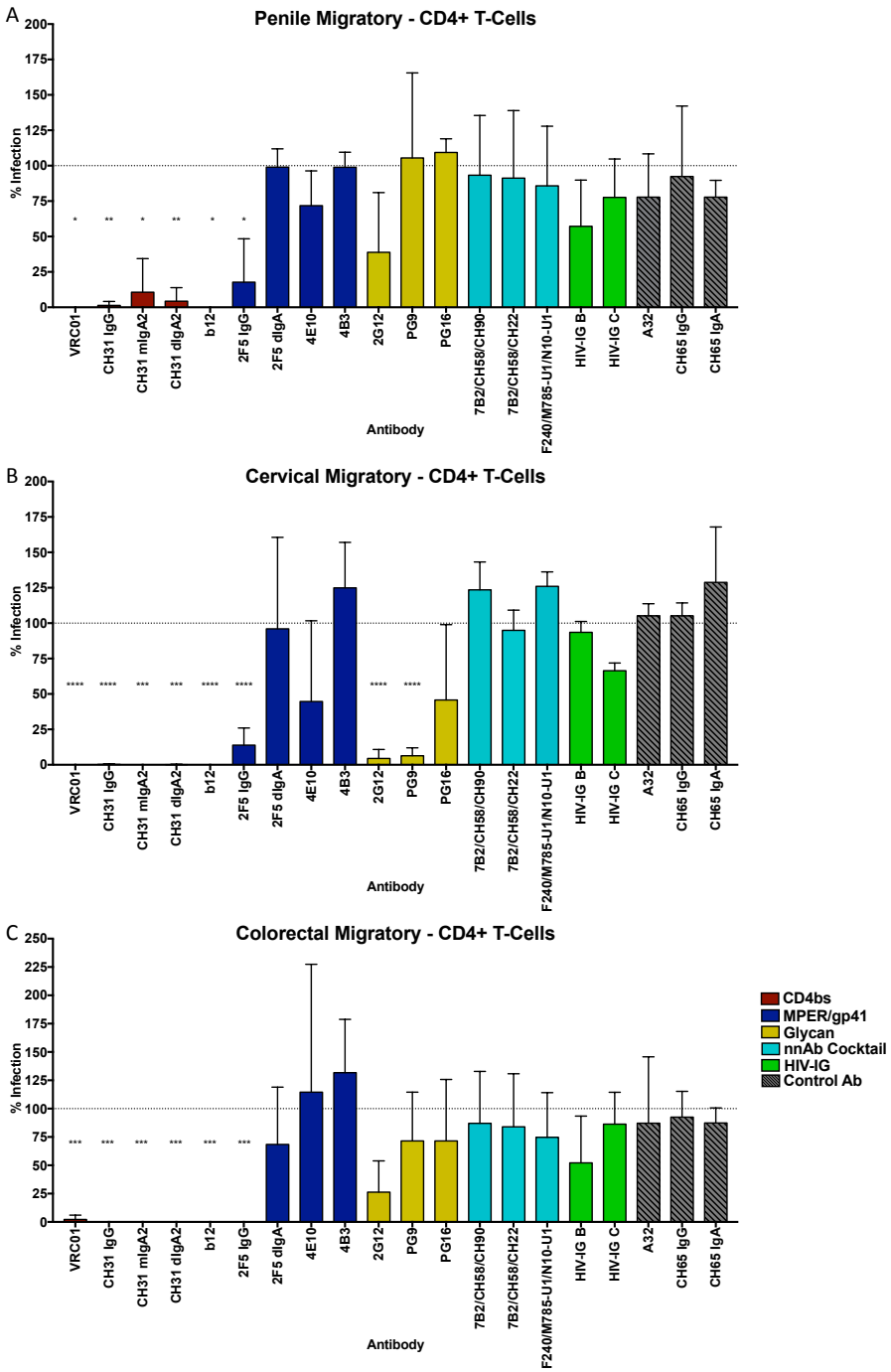
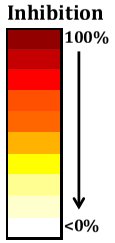


Figure 4.10 Inhibition by single antibodies and antibody cocktails of HIV-1_{BaL} infection from mucosal migratory cells to CD4+ target cells.

Inhibition of HIV-1_{BaL} by antibody panels in preventing the onward infection of CD4+ T cells by migratory cells isolated from (A) penile glans (n=3), (B) ectocervical (n=2 HIV-IG B & C; n=3, remaining Ab) and (C) colorectal tissue (n=3). Data is presented as percentage infection when compared to the HIV-1_{BaL}-positive control. One-way ANOVA with Dunnett's multiple comparison test followed by an unpaired t test was used to compare the antibodies with the CH65 isotype controls. [*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001]

Table 4.5 Summary of percentage inhibition for all inhibition assays

Antibody	Antibody Specificity	Cellular								Tissue						Tissue Migratory Cells-CD4+ T-Cells					
		Macrophages		*Dendritic Cells*		*DC-T Cells*		*PBMC*		*Penile Glans*		*Ectocervical*		*Colorectal*		*Penile Glans*		*Ectocervical*		*Colorectal*	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
VRC01	CD4-binding site	100.0	±0.0	100.0	±0.0	100.0	±0.0	100.0	±0.0	92.5	±12.9	95.0	±5.4	86.0	±12.2	100.0	±0.0	100.0	±0.0	97.8	±3.8
CH31IgG		95.8	±4.0	81.1	±22.6	66.3	±37.2	100.0	±0.0	85.4	±19.3	92.5	±13.7	76.1	±41.9	98.6	±2.7	99.7	±0.4	100.0	±0.0
CH31 mIgA1		99.0	±1.4	82.2	±22.8	52.8	±42.7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
CH31 mIgA2		73.6	±35.0	5.4	±39.4	91.0	±11.8	100.0	±0.0	84.3	±15.7	60.7	±33.9	97.6	±1.7	89.4	±23.8	100.0	±0.0	100.0	±0.0
CH31 dIgA2		74.5	±36.0	23.1	±22.7	91.4	±13.0	100.0	±0.0	78.2	±21.9	62.3	±35.7	69.6	±48.3	95.7	±9.6	99.9	±0.3	100.0	±0.0
b12		99.8	±0.4	100.0	±0.0	100.0	±0.0	98.9	±1.9	100.0	±0.0	100.0	±0.0	47.6	±83.9	100.0	±0.0	100.0	±0.0	100.0	±0.0
2F5 IgG	Membrane-Proximal External Region (MPER)/gp41	100.0	±0.3	100.0	±2.2	100.0	±0.0	100.0	±0.0	91.8	±6.3	84.0	±11.7	94.0	±4.3	82.3	±30.7	86.1	±12.1	100.0	±0.0
2F5 dIgA		-3.7	±22.5	13.0	±31.8	71.9	±26.1	ND	ND	9.3	±46.7	-6.0	±40.9	91.3	±7.7	1.1	±13.0	4.0	±64.6	31.6	±50.6
4E10		99.1	±1.6	100.0	±1.8	100.0	±0.0	100.0	±0.0	43.3	±87.0	30.2	±50.2	91.7	±13.6	28.2	±24.5	55.3	±57.0	-14.6	±112.8
4B3		99.5	±0.7	73.9	±45.3	47.9	±48.7	21.4	±31.1	6.9	±19.3	41.8	±13.3	51.4	±28.8	1.1	±10.6	-24.9	±32.1	-31.7	±47.1
2G12	Glycan	99.9	±0.1	100.0	±0.0	100.0	±0.0	100.0	±0.0	41.8	±8.4	71.0	±14.1	94.3	±6.2	61.2	±42.2	86.4	±16.5	73.7	±27.5
PG9		100.0	±1.3	99.2	±1.8	100.0	±0.0	100.0	±0.0	90.5	±18.7	96.3	±6.1	35.7	±62.2	-5.5	±60.1	95.2	±5.6	28.5	±43.0
PG16		98.1	±4.1	98.1	±3.6	100.0	±0.1	75.0	±22.5	30.4	±37.2	45.5	±25.1	11.9	±83.5	-9.4	±9.6	54.2	±53.2	28.5	±54.2
7B2/CH58/CH90	Various	90.7	±1.0	56.5	±22.8	9.8	±133.2	87.7	±2.1	6.4	±35.1	11.5	±42.2	10.2	±10.4	6.7	±42.1	-23.6	±19.7	12.9	±45.8
7B2/CH58/CH22		95.8	±2.6	76.2	±23.0	30.7	±81.2	82.2	±5.3	49.8	±44.6	32.8	±39.0	45.2	±50.0	8.8	±47.7	5.1	±14.3	16.1	±46.9
F240/M785-U1/N10-U1		97.2	±2.7	100.0	±2.4	-16.0	±70.8	41.7	±16.4	45.3	±30.4	51.3	±43.1	89.7	±1.9	14.2	±42.1	-26.0	±10.2	25.3	±39.4
HIV-IG B	Polyclonal	100.0	N/A	100.0	N/A	14.1	±80.2	91.0	±3.7	62.5	±19.5	52.1	±59.5	96.7	±4.7	42.8	±32.6	6.4	±7.6	47.8	±41.3
HIV-IG C		100.0	N/A	100.0	N/A	89.5	±9.2	94.7	±1.3	59.2	±25.0	65.1	±15.7	76.1	±27.4	22.4	±27.1	33.5	±5.4	13.7	±28.1
A32	CD4i Cluster 1	-9.5	±37.1	-2.4	±34.3	-18.0	±52.0	83.5	±7.8	42.3	±26.8	57.7	±40.5	77.5	±49.2	22.2	±30.5	-5.2	±8.6	12.8	±58.6
CH65 IgG	Flu Haemagglutinin	1.5	±3.0	12.6	±21.7	10.2	±25.9	-17.9	±74.6	0.4	±41.4	9.2	±22.9	-9.3	±74.8	7.7	±44.1	-5.1	±9.1	7.5	±27.0
CH65 IgA		-0.2	±1.1	14.9	±8.2	8.4	±21.6	13.8	±49.3	41.0	±19.7	5.3	±4.6	23.9	±51.0	22.2	±11.8	-28.8	±39.2	12.6	±13.3



Percentage inhibition of single antibodies (50 μg/mL) and antibody cocktails (25 μg/mL, each). Mean and SD values included. Highlighted cells are <2SD. ND = Not Done. N/A = Not available due to n=1.

4.3. Discussion

This chapter set out to investigate the functional activity of nAb and nnAb in a series of cellular and tissue explant models designed to mimic the early events of transmission at mucosal surfaces.

The initial assessment of non- or weakly-neutralising antibodies for inhibition of HIV-1_{BaL} infection in cellular models yielded few antibodies capable of significantly reducing levels of detectable virus across all cellular models. However, a small number of antibodies demonstrated moderate levels of inhibition in at least one of the cellular models. Interestingly, 7B2AAA demonstrated moderate levels of inhibition in the macrophage cellular model, but was ineffective in mIgA2 or dIgA2 isotypes. Indeed, it has been shown that this antibody was ineffective at inhibiting HIV-1 infection in its natural IgG1 form and only gained inhibitory properties once the AAA mutation was introduced in the Fc-region, increasing FcR binding ability and effector function activity [240].

The nnAb, F240, exhibited good levels of protection against direct infection of macrophages and DCs by HIV-1_{BaL}, although this protective efficacy was lost in the *trans*-infection DC-to-CD4+ T cell model. This echoes previous experimental work where F240 exhibited good inhibition of HIV-1_{BaL} infection of macrophages and DCs, although this inhibition was partially abrogated in PBMC infection [166, 445]. The FcR expression data demonstrated that the macrophages and DCs used within the inhibition assays were high FcR expressers, in particular CD32, CD64 and CD89, whereas PBMC FcR expression

was slightly lower, with approximately 16% of the cells negative for all FcR. This suggests the inhibition observed was most likely Fc-mediated.

CD4bs-specific antibodies were the most efficient at inhibiting HIV-1_{BaL} infection across all inhibitory models investigated. In the neutralisation assays, there was little difference observed between the PBMC and TZM-bl inhibition data, suggesting that CD4bs-specific HIV-1 antibody activity was not dependent upon of FcR-engagement. However, *in vivo* NHP models have demonstrated that FcγR-interactions play an important role in extending the half-life of CD4bs antibodies in plasma [446] and also in viral clearance [447]. Furthermore, the neutralising ability of b12 IgG was reduced when FcγR-binding activity was mutated out [385, 437].

A recent study used an alternative analysis method to interpret neutralisation data, where the dose-response slope was measured and related to breadth of HIV-1 neutralisation [448]. The study assessed multiple HIV-1 specific antibodies in TZM-bl neutralisation assays, including CD4bs-specific antibodies VRC01 and CH31 IgG, and identified antibodies with a dose-response curve <1 as being the most potent and broadly neutralising antibodies. The TZM-bl neutralisation assay data obtained in this chapter was not analysed in using this method, however, it is interesting to note that the antibodies they predicted to be the broadest neutralisers are the antibodies that exhibit the greatest inhibition across all inhibition assays used here.

CH31, a VRC01-like antibody, was assayed in IgG1, mIgA1 (for the cellular assays only), mIgA2 and dIgA2 isotype forms. Interestingly, the data indicated that there was no statistical difference in the performance of the CH31 isotypes across the macrophage, PBMC, DC-to-CD4+ T cell and tissue models. However,

within the DC direct infection model, antibody isotypes IgG and IgA1 efficiently blocked infection within this model, whereas the IgA2 isotypes were not able to inhibit infection of HIV-1_{BaL}. While not statistically significant, the inhibition pattern observed in macrophages for the CH31 IgA2 antibodies was similar. The reasons for these differences in inhibition between the CH31 isotypes observed in these two models, particularly IgA1 versus IgA2, are not clear. One possibility is a difference in structural flexibility between IgA1 and IgA2 isotypes, which could potentially confer improved epitope access to IgA1 isotypes [449, 450]. However, this does not explain the inhibitory activity of the CH31 IgA2 antibodies seen in the DC-to-CD4+ T cell and tissue models. Unfortunately, there was insufficient mIgA1 antibody to perform the inhibition assays in the tissue models to enable full isotype comparisons to be made. These differences in isotype efficacy are interesting observations that may warrant further investigation.

HIV-1 can be transmitted via direct infection by cell-free virus and through cell-to-cell contact of productively infected cells. The CD4bs antibodies demonstrated equivalently high levels of inhibitory activity in the direct infection macrophage cellular and tissue models and in also the DC- and tissue migratories-to-CD4+ T cell models. Previous research has shown that VRC01 and other nAb are capable of blocking cell-free and cell-to-cell transmission of lab-adapted and transmitter/founder viruses, although it was noted that VRC01 was not as efficient as other CD4bs-specific antibodies, NIH45-46 and 3BNC60, which were not used in this chapter [253]. However, it is important to note that the investigators used VRC01 at a concentration of 15 μg/mL, whereas it was

used at 50 μg/mL in this chapter, which possibly accounts for its increased efficacy.

The CD4bs-specific antibody data observed in the inhibition assays in this chapter are promising for future clinical trials. Indeed, recent studies using CD4bs-specific HIV-1 antibodies, including VRC01, in passive infusion experiments in NHP and humans have demonstrated excellent efficacy for the antibody at blocking infection and at lowering the viral load of infected individuals [446, 447, 451-453].

Neutralising MPER-specific antibodies, 2F5 and 4E10 and non-neutralising gp41-specific antibody 4B3, showed mixed levels of inhibitory activity in across the models. These antibodies performed well in the PBMC neutralisation assays, but were ineffective in the TZM-bl classical neutralisation experiments, leading to a possible function for the Fc-region in the activity of these antibodies. Indeed, similar experiments using TZM-bl cells engineered to express CD64 demonstrated improved neutralisation activity for MPER-specific antibodies 2F5 and 4E10 by as much as 5000-fold when compared to wild-type TZM-bl cells [454].

Of the MPER-specific nAb, only 2F5 IgG demonstrated inhibitory ability across all cellular and tissue models investigated, consistent with *in vivo* NHP challenge experiments that concluded 2F5 IgG was capable of preventing $SHIV_{BaL}$ infection in a dose-dependent manner [455, 456]. Interestingly, in contrast to the IgG isotype, 2F5 dIgA was unable to inhibit in the majority of inhibition assays tested, which is reflective of previous studies showing the neutralisation ability of 2F5 was reduced in polymeric dIgA and pIgM isotypes

[457, 458]. However, some inhibition was observed in the DC-to-CD4+ T cell and colorectal tissue models.

4E10 demonstrated good levels of inhibition across all the cellular models, with moderate inhibition seen in the colorectal tissue assay. However, negligible inhibition was noted in the penile glans and cervical tissue models, while no inhibition was observed in the tissue migratory cell-to-CD4+ T cell models. NHP studies have been used to show that 4E10 blocks intrarectal challenge of $SHIV_{BaL}$, although the authors note that the half-life of MPER-specific antibodies *in vivo* is relatively short and they therefore had to boost protection through the administration of a second antibody infusion [456].

Gp41-specific nnAb, 4B3, demonstrated good inhibitory activity in the macrophage and DC models, but was unable to significantly inhibit in any of the other inhibition models. This is reflective of data showing that 4B3 has good inhibitory activity in macrophages, with an ability to bind free HIV-1 virions and induce high levels of ADCC, but was not capable of *in vivo* neutralisation in NHP vaginal challenge [166].

The results for the MPER/gp41-specific antibodies suggest that 2F5 and 4E10 IgG could have potential to prevent HIV-1 infection *in vivo*. However, some caution should be used with MPER-specific antibodies as research indicates that, in some individuals, these antibodies can have auto-reactive properties, which could possibly reduce their effectiveness [459]. Additionally, it is important to recognise that the MPER-binding site can be occluded to antibody in more highly resistant HIV-1 isolates [460], while binding to the CD4 target receptor has been shown to decrease the recognition of 2F5 and 4E10 to the MPER-epitope on cleaved HIV-1 envelope spikes [461]. Furthermore, it has been observed that

4E10, although comparatively efficient at blocking direct infection of cell-free virus, was poor at inhibiting cell-to-cell spread of HIV-1 [253].

V1V2 glycan-specific antibodies, PG9 and PG16, and V3 glycan-specific antibody, 2G12, demonstrated good levels of inhibition across all cellular models. However, despite high levels of classical neutralisation in the TZM-bl assay, more variable results were observed in the tissue inhibition assays for PG9 and PG16. PG9 demonstrated good levels of inhibition in penile glans and ectocervical tissue, but inhibited poorly in colorectal tissue, whereas PG16 was poor at inhibiting HIV-1_{BaL} infection across all tissue models. Within the migratory-to-CD4+ T cell assays, PG9 demonstrated good levels of inhibition in the ectocervical migratory cell assay, but was a poor inhibitor in the other two tissue migratory cell models, whereas PG16 did not inhibit infection in any of the migratory models. This is partially reflective of a similar *ex vivo* study, which used colorectal and ectocervical tissue explants to investigate the activity of PG9 and PG16 against HIV-1_{JR-CSF} and demonstrated that levels of virus were continuously inhibited within the ectocervical tissue model, but viral control was lost within the colorectal tissue models after 21 days in culture [462]. However, it should be noted that the authors used 50-fold higher antibody concentration on the ectocervical tissue, which could account for this sustained inhibitory activity.

Interestingly, with the exception of the penile glans tissue explant model, 2G12 performed well across all inhibition assays and exerted similar levels of inhibition in the migratory cell assays for the tissue types tested, although it performed poorly in the classical neutralisation assay on TZM-bl cells. 2G12 has previously been shown to strongly bind to CD16 and moderately bind to CD32

[166], which could explain the lack of inhibition seen in the TZM-bl neutralisation assay. Moreover, *in vivo* passive infusion experiments have demonstrated that 2G12 exerts potent inhibitory activity within NHP models, which was not correlated to its activity within classical neutralisation assays [463]. 2G12 recognises epitopes exclusively at positions N295, N332, N394, N386, N448 in the HIV-1 glycan shield [464, 465].

While the glycan-specific antibodies generally performed well, it is important to note that, due to heterogeneity in glycosylation of HIV-1 Env, glycan-specific antibodies are not able to neutralise 100% of virus within assays [466, 467]. This leaves these antibodies vulnerable to virus escape, which could explain some of the results obtained within this chapter, especially for the longer, 21 day, tissue explant experiments. Furthermore, underglycosylation of transmitter/founder viruses has been reported, leading to an inability of glycan-specific antibodies to bind [468, 469].

NnAb exert their inhibitory activity through FcR-engagement; therefore, it was not surprising that the nnAb cocktails antibodies demonstrated little activity in the classical neutralisation assay using TZM-bl indicator cells, whereas moderate activity was observed in the PBMC assay.

Within the cellular inhibition assays, the nnAb cocktails showed good levels of inhibition in the macrophage and DC models. The FcR expression data demonstrated that both of the cell subsets used within these assays expressed high levels of FcR. Both macrophages and DCs can mediate ADCP, ADCC and ADCVI (reviewed in [101]), while previous *ex vivo* experiments using 7B2, CH22 and F240, have demonstrated that these antibodies bind well to FcR and inhibit

within macrophage and DC models [240, 445]. Therefore, it can be assumed that the modes of inhibition within these models are FcR-dependent.

Within the DC-to-CD4+ T cell model, the nnAb were not capable of preventing the onward transmission of HIV-1_{BaL}. Here, DCs were co-incubated with HIV-1_{BaL} for 1 hour to allow for virus capture, but not productive infection; therefore, the mode of transmission was predominantly via *trans*-infection. Cell-to-cell transmission of HIV-1 is extremely efficient, with mathematical modelling suggesting that it may be 100-1000 times faster than cell-free transfer [252]. Indeed, research investigating the ability of DCs to transfer HIV-1 to CD4+ T cell has shown they efficiently transmit the virus via both *cis*- and *trans*-infection [68]. As previously mentioned, studies investigating the abilities of bnAb to block cell-to-cell transmission of HIV-1 have shown that the efficacy of some antibodies is reduced when compared to blocking of cell-free virus [253, 470]. Therefore, it is possible that nnAb are not able to block cell-to-cell transmission of HIV-1.

Within the tissue explant models, the nnAb were not able to significantly inhibit the direct infection of tissue. The data obtained in Chapter 3 indicated that FcR-expression was low across all three mucosal tissues, which is of potential relevance to the results obtained here, as there may be too few effector cells and associated FcR expressed to enable sufficient inhibitory immune responses to block infection. Similar to the results observed in DC-to-CD4+ T cell model, the antibody cocktails showed no inhibitory ability in blocking onward infection in any of the migratory tissue cell models.

However, it should be noted that the F240/M785-U1/N10-U1 antibody cocktail exerted a mean inhibitory value of 89.7% within the colorectal tissue

model. The results from Chapter 3 demonstrated that this tissue type had the greatest percentage expression of CD14+ monocytic cells. While the absolute numbers of cells were not enumerated within this study, it is possible that they are sufficient to reduce HIV-1_{BaL} infection within the colorectal tissue. However, given that the antibody activity was lost in the migratory-to-CD4+ T cell model for this tissue type, it may be that this activity would not be sufficient to prevent the onward transmission of virus, *in vivo*.

Although there was no statistically significant inhibition seen within the mucosal tissue explant models, the nnAb cocktail 7B2/CH58/CH22 demonstrated a trend toward greater levels of inhibition when compared to the 7B2/CH58/CH90 cocktail across the three tissue types. Recent i*n vivo* experiments investigated the ability of intravenous passive infusion of 7B2AAA, CH22AAA and A32 to prevent $SHIV_{BaL}$ infection in Indian-origin Rhesus monkeys post-intrarectal challenge. The results demonstrated that nnAb 7B2AAA and A32 had no ability to block infection, while CH22 prevented infection in 4 out of 6 animals challenged [240].

Although several investigations have used nnAb in passive infusion experiments in NHP, none have successfully demonstrated an ability to block HIV/SHIV transmission [166, 239, 240, 428]. However, several groups have suggested that nnAb have an ability to reduce the number of transmitter/founder viruses [240] and reduced viral set point post-infection [166], indicating that nnAb could have a role in the final outcome of HIV-1 transmission.

One important caveat is that the majority of this study uses monoclonal antibodies, with a maximum combination of three different mAb used in the

nnAb cocktails. HIV-IGs are polyclonal in nature, potentially conferring an advantage over monoclonal antibodies through the ability to target multiple HIV-1 epitopes. Here, clade B- and clade C-specific HIV-IGs demonstrated good inhibition in the macrophage, DC and PBMC models, while HIV-IG C reduced HIV-1_{BaL} infection in the DC-to-CD4+ T cell model. Within the mucosal explant models, both HIV-IG B and C exerted a limited amount of inhibitory function, irrespective of mucosal tissue type, while no inhibition was noted in the tissue migratory-to-CD4+ T cell models. Historically, passive IV-IG therapy has been used effectively in the prevention of a range of infectious viral diseases including Hepatitis A and B, Measles, Rabies and Ebola (reviewed in [471, 472]), although efficacy trials investigating the use of HIV-1 specific immunoglobulin therapy in preventing MTCT [473] or for post-infection treatment proved unsuccessful [474]. Similar to the HIV-IG passive infusion trials in humans, NHP studies have demonstrated no efficacy with polyclonal HIV-IG (isolated from elite controllers) [239], SIV-IG [428] or SHIV-IG [475] in blocking virus transmission. However, the SIV-IG and SHIV-IG studies highlighted the potential for the polyclonal antibodies to bind HIV-1 Gag, Tat and Rev of different clades and commented that the neutralisation activity seen in PBMC assays was reduced 20-fold after depletion of NK cells, suggesting SHIV-IGs exert inhibitory activity through Fc-FcR interactions and effector functions. HIV-IGs have also been shown to bind a fraction of infectious HIV-1_{BaL} virions, which could be of relevance when interpreting in the inhibition conferred by HIV-IGs in this chapter [476].

While the relative composition of subclasses for the HIV-IGs used in this chapter were not determined, it is important to note that all monoclonal antibodies used here were IgG1, whereas the HIV-IGs were a composite of all

subclasses, which could potentially impact their effector function activity. Of relevance is a study that dissected the comparative effectiveness of the individual HIV-IG IgG subclasses at blocking HIV infection *in vitro* and found HIV-IG IgG3 was more potent when compared to HIV-IG IgG1 or IgG2 [477]. Although the investigators attributed the difference in inhibition to the more flexible hinge region found on IgG3 subclasses, other studies have shown that IgG3 is an efficient inducer of antibody-dependent effector functions [133].

However, given the overall results determined in this study and the combined historical use of HIV-IGs by the wider HIV field, it seems that a more specific, monoclonal antibody approach may be more effective at preventing HIV-1 acquisition than a generic polyclonal antibody approach.

Due to limited antibody availability, it was only possible to investigate the activity of antibodies in inhibiting infection of the clade B laboratory isolate HIV-1_{BaL} throughout. It would be of interest to investigate the activity of antibodies using transmitter/founder isolates and a wider range of viral clades.

NnAb demonstrated greater variability across all tissue assays. As discussed in Chapter 3, FcR expression can be influenced by a number of factors including cytokine milieu, infectious diseases [421, 422], diet, antibiotic use [417] and hormonal changes [266, 267, 416]. In addition, FcR genetic variation can alter the outcome of Fc-FcR interactions, a factor that has been shown to impact on MTCT of HIV-1 [478, 479]. Combined, these influences could account for the wider variability in data observed for the nnAb within the assays used in this study.

Overall, this chapter sought to determine the ability of a range of nAb and nnAb to inhibit infection of HIV-1_{BaL} in cellular and tissue explant models. To summarise, the data showed:

- CD4bs-specific nAb consistently demonstrated high levels of inhibition of HIV-1_{BaL} across all cellular and tissue models.
- MPER- and glycan-specific nAb demonstrated high levels of inhibition in the cellular models, but were less efficient at inhibiting HIV-1_{BaL} in the tissue models.
- NnAb cocktails demonstrated high levels of inhibition in the macrophage and DCs models, but this inhibitory effect was lost in the DC-to-CD4+ T cell model. Furthermore, in the tissue and migratory-to-CD4+ T cells models, the nnAb cocktails showed limited ability to inhibit HIV-1_{BaL} infection.
- Polyclonal HIV-IG antibodies demonstrated similar patterns of inhibition to those seen with nnAb cocktails.

The combination of higher FcR expression on macrophages and DCs and good levels of antibody inhibition irrespective of conventional antibody neutralisation ability demonstrate that these cell types have interesting potential that will be further investigated in Chapter 5. The previous results chapter determined the FcR profile of cells isolated from mucosal tissue and demonstrated that a large proportion of effector cells within mucosal tissue are negative for CD16, CD32, CD64 and CD89. However, it is of importance to note that these surfaces are associated with high circulatory blood flow, which could potentially impact on the inhibitory activity conferred by the nnAb. Infiltrating cells from the whole blood are a composite of innate and adaptive immune

leucocytes. Of interest in the context of this thesis, are the high numbers of immune effector cells such as neutrophils, NK cells, macrophages and DCs, all capable of FcR-dependent cellular effector functions. This will be further explored in Chapter 5 using an experiment designed to mimic infiltrating whole blood and assess the relative impact of peripheral effector cells on the activity of nAb and nnAb in inhibiting HIV-1_{BaL} infection.

CHAPTER 5 – RESULTS:

Whole Blood Assay to Assess Antiviral Function of HIV-1 Specific Antibodies

5. Whole Blood Assay to Assess Antiviral Function of HIV-1 Specific Antibodies

5.1. Introduction

HIV-1 transmission occurs most frequently at mucosal surfaces through sexual intercourse. During the very early stages of infection, a small foci of infected target cells quickly leads to systemic dissemination of the virus through inflammatory signals attracting fresh target cells to the area and also dendritic cell tracking to the lymph nodes, fuelling interaction with target cells [44, 246]. While cells from the periphery are known to play a role in viral dissemination, they also have potential to enhance Fc-mediated effector functions in the presence of HIV-1 antibodies.

Fc-mediated effector functions, such as ADCC, ADCP and ADCVI are initiated through cross-linking of FcR. These receptors are found on the surface of NK cells, macrophages, DCs and neutrophils, the latter being the most abundant leucocyte within whole blood, rapidly recruited to sites of inflammation [118]. Once HIV-1 has gained entry through mucosal epithelium and established a foci of infection, NHP studies have shown an increased expression of chemokines, such as CCL3, CCL20 and CXCL8 [480]. In turn, these chemokines are thought to drive the recruitment of DCs, macrophages and NK cells to the mucosal site of infection [480, 481]. Therefore, these cells have the potential to mediate Fc effector functions that may be key to an effective immune response during the early stages of HIV-1 infection at mucosal surfaces.

In addition to antibody-mediated cellular effector functions, complement activity should also be considered at the early stages of HIV-1 infection. The

complement cascade acts through one of three pathways; the classical, alternative or MBL pathway, each culminating in CDC and the death of the target cell. The complement pathways have the potential to impact on HIV-1 infection, although their exact roles during early infection remain unclear. While studies have demonstrated that complement may have a protective role in early HIV-1 infection [123, 124] and also in elite controllers [126], the virus has evolved a number of mechanisms to evade complement activity and may potentially use the complement system to enhance infectivity of target cells [127-129]. Of particular interest in the context of antibody-mediated HIV-1 activity is the classical complement pathway, which is activated through complement components binding with high avidity to the Fc-portion of an antibody-opsonised antigen or infected cell [125].

Fc-mediated effector functions also play an important role during the course of natural infection. ADCC-inducing antibodies can lower the viral set-point in NHP models [166-169], while studies in humans indicate that ADCC antibodies correlate with slower disease progression [167, 170] and significantly lower genital HIV-1 RNA levels in seropositive women [171]. In addition, high ADCVI activity in vaccinated individuals inversely correlates with lower rates of sexually acquired HIV-1 infection [179]. Furthermore, the RV144 trial showed an efficacy of 31.2%, despite the lack of nAb and limited CD8+ T cell responses [353, 440]. Subsequent correlates of protection analysis demonstrated a clear positive association between levels of non-neutralising IgG3 antibodies to the gp120 variable regions V1V2 of the envelope glycoprotein structure, with high ADCC activity and a reduced risk of infection. The correlated protection was reduced in the presence of IgA to the same region of gp120 [134,

354, 355, 358, 375]. Finally, while nnAb act through Fc-mediated effector functions, it is important to remember that nAb are also capable of engaging FcR, which further enhances the neutralising activity of these antibodies [236, 482, 483].

The plethora of cells and components capable of effector functions found within whole blood means that it is important for an assay system to contain all elements of the immune system. At present, the majority of assays used to investigate ADCC and ADCP require isolation of a particular cell subset from PBMC, i.e. NK cells, neutrophils or macrophages. Moreover, *in vitro* assays further expand the purified effector cell populations to setup an effector:target ratio that is optimised to demonstrate a biological effect. This may be far removed from the natural *in vivo* context, whereby infiltrating cells will not only contain a mixture of immune effector cells, but will also contain complement and other blood-borne factors. These immune cells or factors are capable of antibody-dependent functions, which could work in cooperation with one another, so it is more relevant that any assay used to determine their function contains a combination of all components. Furthermore, with immune cells from the whole blood known to infiltrate mucosal tissue in response to early HIV-1 infection, their antiviral response to HIV-1 in the presence of antibodies (both nAb and nnAb) could be important. However, if antibodies show minimal activity within a whole blood environment, their ability to prevent HIV-1 transmission at mucosal sites is likely to be negligible.

This thesis has so far sought to determine the expression of FcR and availability of immune effector cells within penile glans, ectocervical and colorectal tissue, demonstrating in Chapter 3 that FcR expression and immune

cells are found at significantly lower levels when compared to cells isolated from the whole blood. Furthermore, investigations into the activity of nAb and nnAb within the mucosal tissues in Chapter 4 revealed that nAb had the greatest inhibitory activity within these *in vitro* assay systems. However, it is important to note that these assay systems are isolated and devoid of infiltrating cells from the periphery. This chapter attempts to determine whether whole blood has the ability to induce antibody-dependent cellular immune responses to HIV-1 that differ from those seen in mucosal tissues. In addition, results from this chapter will help inform on whether peripheral immune cells with the potential to infiltrate mucosal tissues have the ability to reduce HIV-1 infection. As such, an assay has been developed that allows for assessment of antibody activity in the presence or absence of whole blood, as depicted in Figure 5.1.

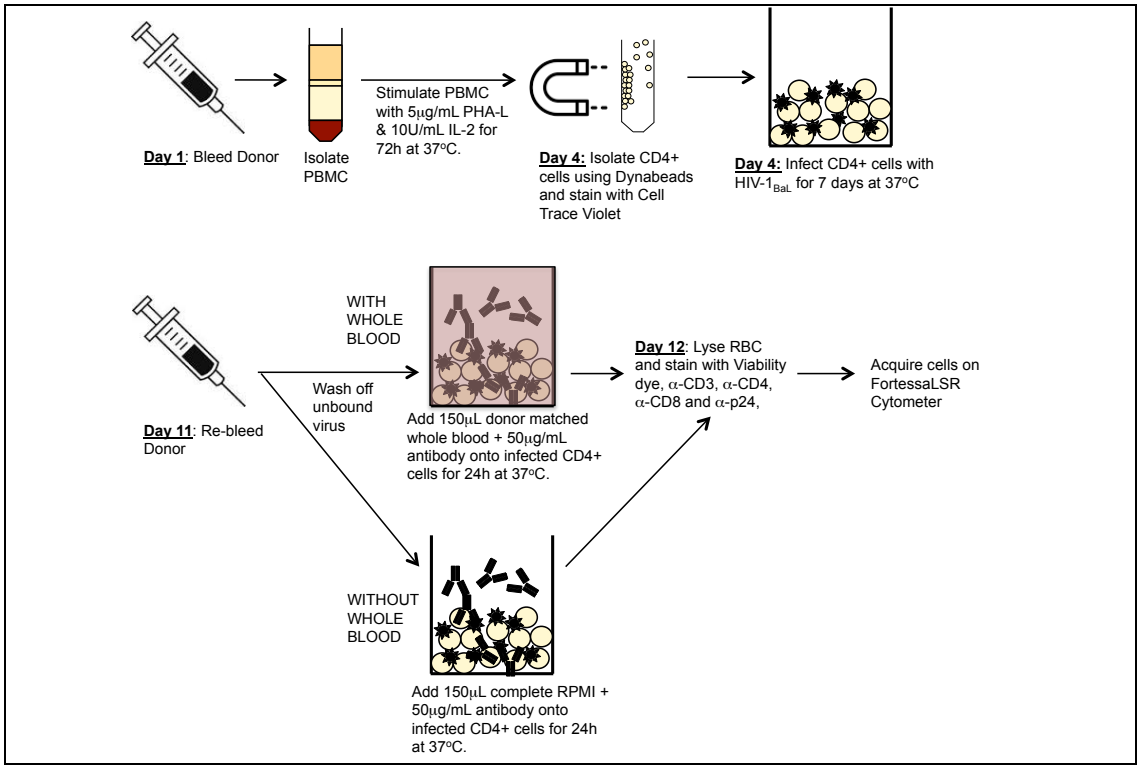


Figure 5.1 Schematic of whole blood assay setup.

Experimental design for the whole blood assay. NB. For the initial screening experiments, cell trace violet (CTV) was not added at day 4.

The aims of this chapter are to:

1. Develop a whole blood assay to assess antibody-induced antiviral activity.
2. Assess the antiviral activity of nAb and nnAb against HIV-1 infected CD4+ cells in the presence or absence of whole blood.
3. Assess the binding of nAb and nnAb to HIV-1 infected CD4+ T cells *in vitro*.
4. Assess the ability of nAb and nnAb to prevent onward cell-to-cell infection within whole blood cultures.

5.2. Results

5.2.1. HIV-1_{BaL} Titration and Experimental Setup

As an initial step in establishing the whole blood assay, experiments were performed to determine the appropriate concentration of virus (HIV-1_{BaL}) and length of incubation required to generate a sufficient population of p24 antigen positive CD4+ cells by flow cytometry. As such, CD4+ cells were isolated from PBMC and stimulated for 72 hours with 5 μg/mL PHA-L and 10 U/mL IL-2 before being exposed to HIV-1_{BaL} at $TCID_{50}$ $2x10^3$, $1x10^3$ or $1x10^2$ for 3, 5 or 7 days (Figure 5.2). The data were analysed using a flow cytometry panel to assess the percentage p24+ CD4+ cells within the total population. Previous studies have demonstrated that 40% p24+CD4+ cells in the total viable population is an acceptable percentage to enable adequate assessment of antiviral antibody activity via flow cytometry [484, 485]. The results here indicate that the optimal conditions to achieve at least 40% p24+CD4+ cells were a $TCID_{50}$ of $2x10^3$ and an incubation period of 7 days (Figure 5.3). This concentration of virus and length of incubation was used for all subsequent experiments.

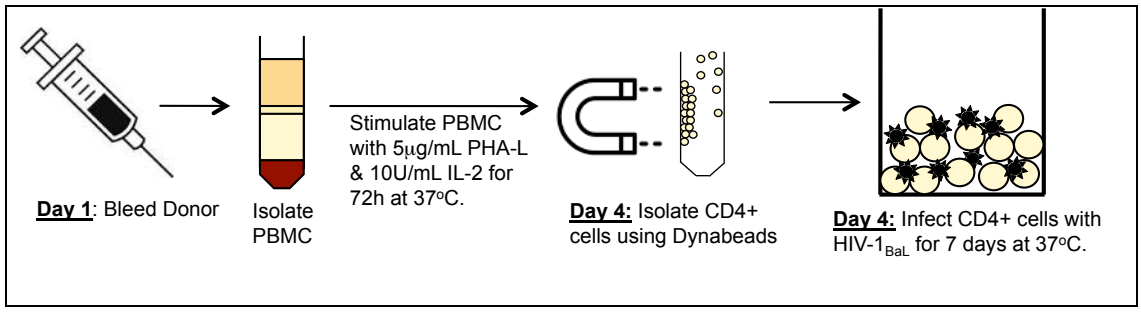


Figure 5.2 Schematic for the isolation and infection of CD4+ cells.

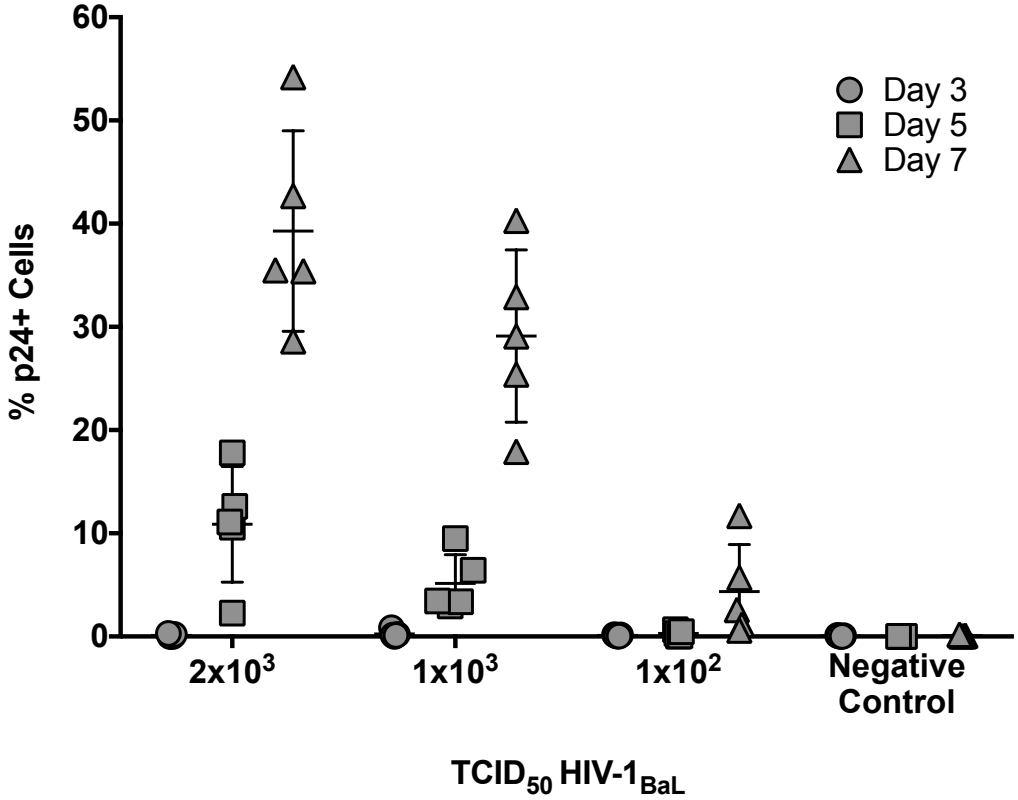


Figure 5.3 HIV-1_{BaL} titration and time course experiment

Assessment of viral infectivity was determined using CD4+ cells (n=5), incubated with HIV-1_{BaL} at $2x10^3$, $1x10^3$ or $1x10^2$ $TCID_{50}$ for 3, 5 or 7 days.

5.2.2. Impact of Whole Blood on Antibody Antiviral Function

During the course of infection at mucosal surfaces, inflammatory signals could potentially lead to an influx of immune cells, such as NK cells, DCs, monocytes, macrophages and neutrophils, from the periphery. Here, an *in vitro* whole blood assay was designed to investigate the potential impact of these peripheral cells on the ability of nAb and nnAb to reduce HIV-1_{BaL} infection (Figure 5.1). The initial experiment sought to determine any difference between the levels of infection, as determined by the frequency of p24+ cells, with or without the addition of whole blood to the assay system.

CD4bs-specific antibodies, VRC01, CH31 and b12 reduced the percentage of p24+ cells when compared to the infected control, irrespective of the addition of whole blood to the system. Without whole blood, VRC01 reduced the percentage of p24+ cells by 63.5% [****P<0.0001] (Figure 5.4A), whereas with whole blood, this reduction was 50.5% [** P<0.01] (Figure 5.4B). Similarly, CH31 reduced the percentage of p24+ cells by 47.0% [**P<0.01] (Figure 5.4A) without whole blood and 57.0% [**P<0.01] (Figure 5.4B) with whole blood, whilst b12 reduced p24+ cells by 57.6% [***P<0.001] (Figure 5.4A) without whole blood and 58.0% [**P<0.01] (Figure 5.4B) with whole blood.

MPER-specific antibodies, 2F5 and 4E10 showed no significant ability to reduce p24+ cells when compared to the infected control in either of the assay models (Figure 5.4).

Of the glycan-specific antibodies, PG9 and PG16 demonstrated significant reduction of p24+ cells without whole blood when compared to the infected control, 61.4% [****P<0.0001] and 42.3% [**P<0.01], respectively, whereas no significant reduction was observed for 2G12 (Figure 5.4A). With the addition of

whole blood, only PG9 showed a significant reduction in p24+ cells, 54.2% [**P<0.01] (Figure 5.4B).

No significant reduction in the percentage of p24+ cells was observed for the nnAb antibody cocktails without whole blood (Figure 5.4A). However, with the addition of whole blood, p24+ cells were significantly reduced by 41.2% [*P<0.05; 7B2/CH58/CH90], 45.8% [*P<0.05; 7B2/CH58/CH22] and 53.9% [**P<0.01; F240/M785-U1/N10-U1] (Figure 5.4B).

Single nnAb, 17b and A32, were used as control antibodies for their ability to bind CD4-inducible epitopes and induce ADCC. However, no reduction in p24+ cells was observed for either antibody, irrespective of the addition of whole blood (Figure 5.4).

However, the assay format does not distinguish between whether the p24+ cells were from the original population of CD4+ cells incubated with virus on day 4 of the assay or whether they are CD4+ cells from whole blood, added on day 11. Subsequent experiments were performed to determine whether percentage p24+ cells were reduced by antibodies targeting productively infected cells or if antibodies were preventing onward transmission of HIV-1_{BaL} to fresh CD4+ T cells introduced by the addition of whole blood.

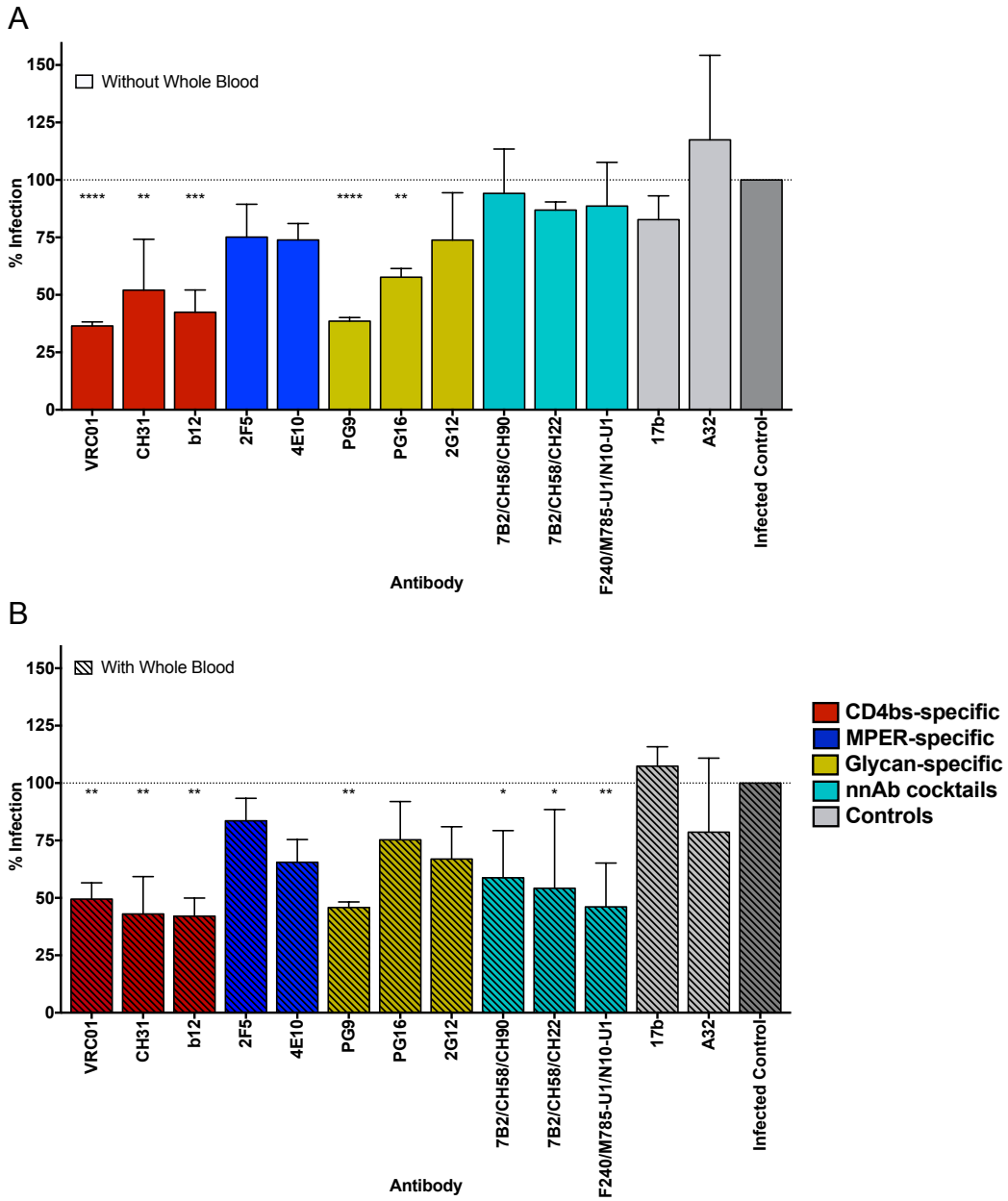


Figure 5.4 Whole Blood Assay antibody screen

The ability of nAb and nnAb (50 μg/mL or 25 μg/mL, each (cocktails)) to reduce levels of HIV-1_{BaL} infection (A) without the addition of whole blood (n=3) or (B) with the addition of whole blood (n=3). Statistical analysis performed using two-way ANOVA with Dunnett's multiple comparison correction. [*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001].

5.2.3. Reduction of p24+CD4+ Cells Cultured with HIV-1 for 7 days

To further investigate the inhibitory activities observed in the whole blood assay, a cell trace violet stain was added to the CD4+ cells on the day of infection to allow for discrimination between the original CD4+ cells and CD4+ cells contained within the supplemented whole blood. As before, nAb and nnAb were added at the same time as the whole blood (Figure 5.1). This allowed for direct assessment of reduction in percentage of p24+ cells in the original CD4+ cell (CTV+) population without inclusion of CD4+ cells in whole blood added to the culture for the last 24 hours (CTV-). The results were determined using flow cytometry and the gating strategy outlined in Figure 5.5 was applied.

Addition of the CD4bs-specific antibody, b12, provided a significant reduction in CD4+CTV+p24+ cells [**P<0.01] when compared to the infected control. A similar trend was observed for VRC01 and CH31, although they did not reach significance (Figure 5.6).

The MPER-specific antibodies, 2F5 and 4E10 demonstrated no reduction in the CD4+CTV+p24+ cell subset (Figure 5.6). Similarly, the glycan-specific, nnAb cocktails and single nnAb showed no ability to reduce the percentage of CD4+CTV+p24+ cells compared to the infected control (Figure 5.6).

Interestingly, the results here show a similar pattern of reduction to that seen in Figure 5.4A, suggesting that the addition of whole blood to the assay system may not be contributing to the reduction in p24+ cells observed for the CD4bs-specific antibodies over the 24 h culture period.

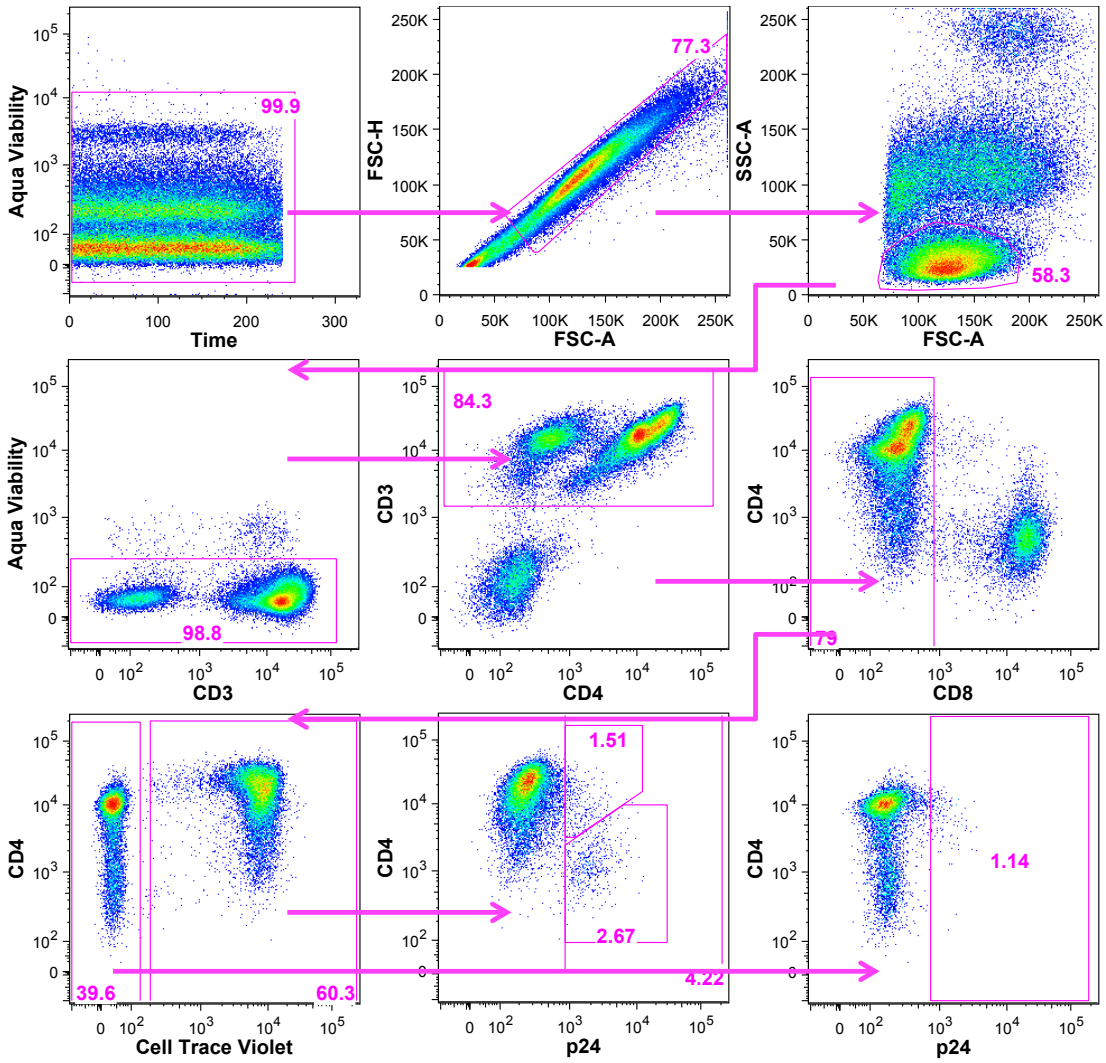


Figure 5.5 Whole blood assay flow cytometry gating strategy

To analyse the data a time gate was first applied to exclude any electronic noise. Following this, a singlet gate excluded any doublets and a lymphocyte gate ensured only cells of interest were analysed. Next, a viability gate excluded any dead cells. CD3+ cells were then selected, followed by CD8- cells. CD3+CD8- cells were divided into cell trace violet (CTV) positive and negative and the amount of p24 expression was investigated. (Data shows representative plots for CD4+ cells incubated with HIV-1_{BaL} for 7days, followed by the addition of 150 μL blood for a further 24 hour incubation.)

NB. The additional gates in the CTV+ population distinguish between the $CD4^{hi}p24^{lo}$ and $CD4^{lo}p24^{hi}$ cell subsets used in the antibody binding experiments,

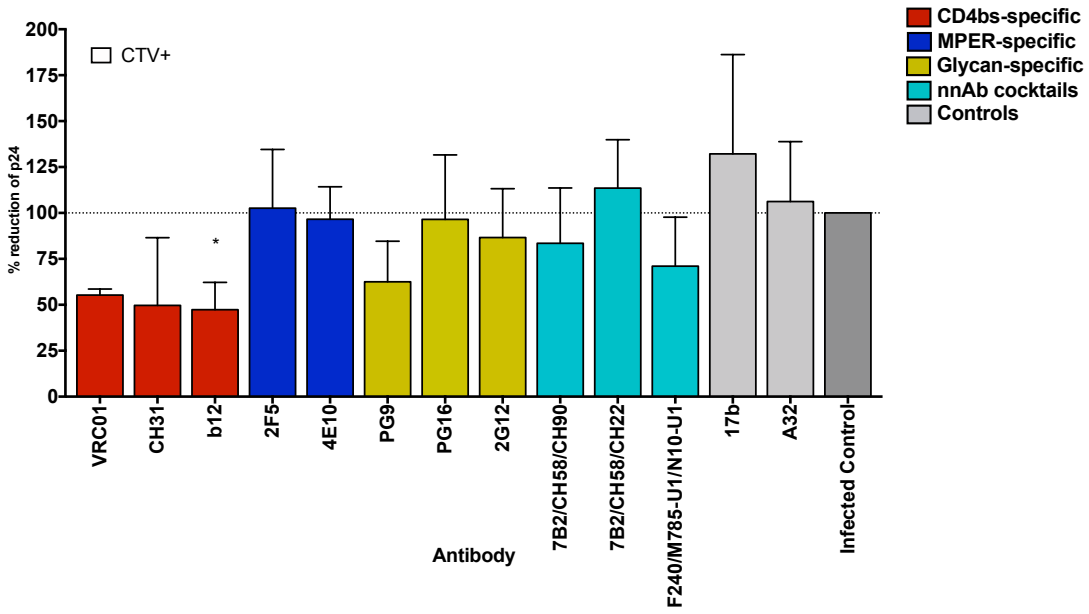


Figure 5.6 Antibody-induced reduction of p24+ cells.

The ability of nAb and nnAb (50 μg/mL or 25 μg/mL, each (cocktails)) to reduce levels of HIV-1_{BaL} p24 with the addition of whole blood in CD4+ cell trace violet (CTV+) positive cells, productively infected with HIV-1_{BaL} for 7 days (n=3). Graph shows percentage p24 reduction in relation to the infected, media-only control. Statistical analysis performed using two-way ANOVA with Dunnett's multiple comparison correction. [*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001].

5.2.4. Comparison of HIV-1 Specific Antibody Binding to CD4+ Cells

During HIV-1 infection, CD4 is downregulated, which is indicative of a cell that is productively infected with the virus. To investigate whether the antibodies used throughout these experiments had a propensity to bind to productively infected cells or newly infected/virally captured CD4+ cells, the experimental design described below was employed (Figure 5.7).

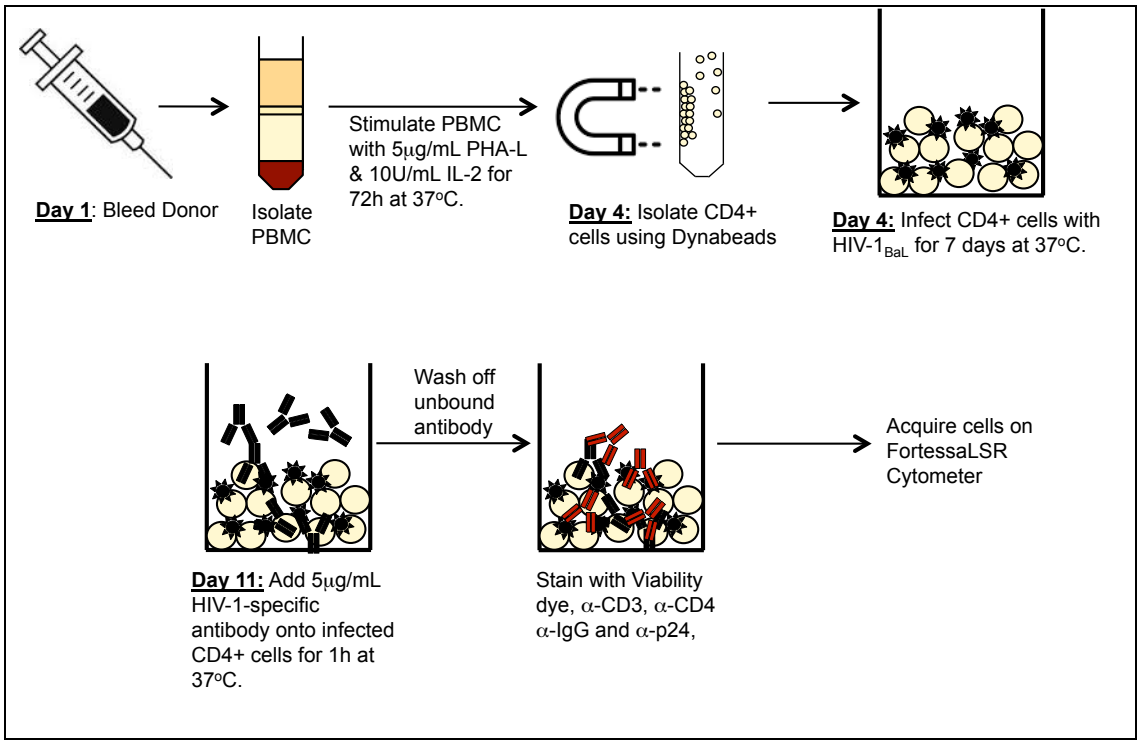


Figure 5.7 Schematic of the antibody binding experiment

CD4+ cells were isolated and infected with HIV-1_{BaL} for 7 days. HIV-1 specific antibodies were incubated with the cells at 5 μg/mL and anti-IgG-APC used to investigate the amount of antibody binding using flow cytometry. Antibody binding to CD4 cell subsets was determined based on the expression of CD4 and p24 markers.

The analysis focused on $CD4^{hi}p24^{lo}$, $CD4^{lo}p24^{hi}$ or CD4+p24- populations, as an indicator of level of HIV-1_{BaL} infection (Figure 5.5). As all antibodies were IgG1, relative binding to the distinct populations could be determined.

Three main antibody profiles were observed (Figure 5.8): a) preferential binding to $CD4^{lo}p24^{hi}$ cells; b) preferential binding to $CD4^{hi}p24^{lo}$ cells; c) preferential binding to CD4+p24- cells. Antibodies falling into category a) included VRC01, CH31, b12, 2F5, PG9 and PG16. Antibodies in category b) included 17b, A32 and the two 7B2 antibody cocktails. Lastly, antibodies in category c) included the F240 nnAb cocktail. 4E10 and 2G12 were exceptions, with 4E10 binding equally to populations a) and b) and 2G12 binding similarly to populations b) and c).

These data are corroborated by analysis of mean fluorescent intensity (MFI) plots for CD4+p24-, $CD4^{hi}$ $p24^{lo}$ and $CD4^{lo}$ $p24^{hi}$ populations. These display the three main categories. Antibodies in category a) displayed preferential binding to $CD4^{lo}$ $p24^{hi}$ cells, including VRC01, CH31, b12, 2F5, PG9 and PG16, with low background levels of binding to CD4+ p24- and $CD4^{hi}$ $p24^{lo}$ cells. Antibodies in category b), particularly 2G12, 17b, A32 displayed high level of binding to CD4+ p24- cells relative to those in category a) while the three nnAb cocktails displayed levels of binding to CD4+ p24- cells that were higher than category a) antibodies but lower than those of 2G12, 17b, A32 (Figures 5.9-5.13).

The CD4bs- (Figure 5.9), MPER- (Figure 5.10) and V1V2-glycan-specific (Figure 5.11) antibodies demonstrated similar binding MFIs, whereby the MFI of CD4+p24- and $CD4^{hi}p24^{lo}$ cells were analogous, but there was an increased MFI for $CD4^{lo}p24^{hi}$ cells, indicating that the antibodies were binding to these cells in increased numbers.

2G12 binding showed increased MFI for the CD4+p24- and $CD4^{hi}p24^{lo}$ populations compared to the $CD4^{lo}p24^{hi}$, which was not observed for the other nAb tested (Figure 5.11). Similarly, this increased MFI was observed for the

nnAb cocktails (Figure 5.12) and the single nnAb (Figure 5.13), although there was greater variability in the antibody binding MFI across the donors for the nnAb. Direct comparison of non-specific binding to HIV-1 negative cells was not made for these antibodies. Future work needs to include these controls to allow for a more defined comparison of IgG binding.

The high endogenous level of binding to CD4+ p24- cells is a significant potential confounder when interpreting the binding patterns for category b) antibodies. This is clearly illustrated by the binding patterns for 2G12. When assessing the total population of cells by MFI for 2G12 there is an apparent drop in the staining for $CD4^{lo}$ $p24^{hi}$ population and increase in staining for $CD4^{hi}$ $p24^{lo}$ relative to CD4+ p24- cells (Figure 5.11). However, if using the binding of 2G12 to CD4+ p24- cells (red plot) as a "gating parameter", a significant tail of staining to the right (blue plot) can be observed with respect to the binding to $CD4^{lo}$ $p24^{hi}$ cells. Traditionally, these cells would be taken as the population showing specific staining for Env positive cells. Nevertheless, when considering antibody function such as potential engagement of ADCC the relative proportion of antibodies bound to the surface of the cells irrespective of their specificity may have greater significance. Similar complex patterns were observed for A32 and 17b and the antibody combinations (Figure 5.13).

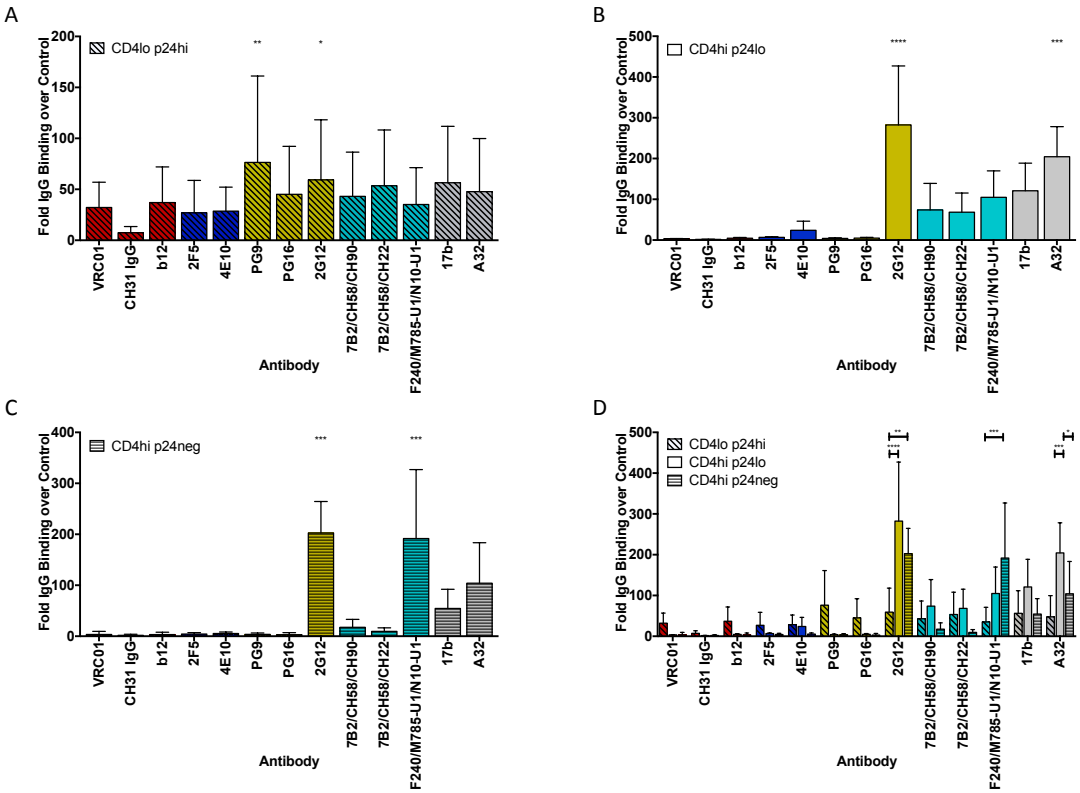


Figure 5.8 Antibody binding to CD4+ cells infected with/exposed to HIV-1_{BaL}.

The ability of nAb and nnAb (5 μg/mL or 5 μg/mL, each (cocktails)) to bind to CD4+ cells infected with HIV-1_{BaL} for 7 days that (A) exhibited downregulated CD4 and high levels of p24 expression (n=3), (B) high CD4 and low levels of p24 expression (n=3), (C) high CD4 expression and negative p24 and (D) a comparison of the IgG binding observed between the (A), (B) and (C). Graphs show fold increase in IgG binding in relation to the infected, media-only control. (A) and (B) statistical analysis performed using two-way ANOVA with Dunnett's multiple comparison correction. (D) statistical analysis performed using multiple t-tests with Holm-Sidak multiple comparison test [*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001].

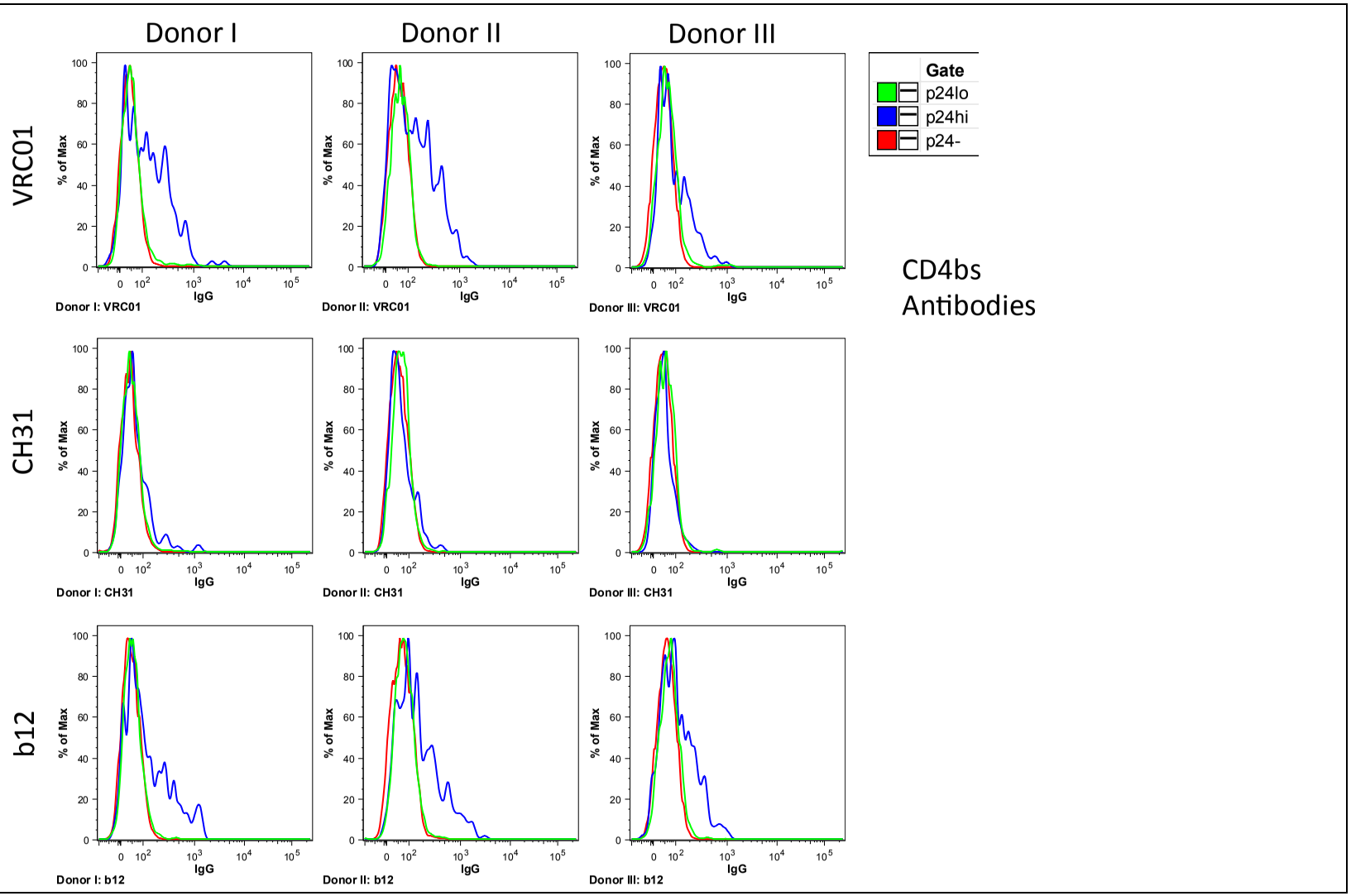


Figure 5.9 CD4bs-specific antibody binding

Histograms indicating MFI of 5 μg/mL IgG-specific binding to $CD4^{hi}p24^{lo}$ (green), $CD4^{lo}p24^{hi}$ (blue) and CD4+ p24- (red) cells infected with HIV-1_{BaL} for 7 days.

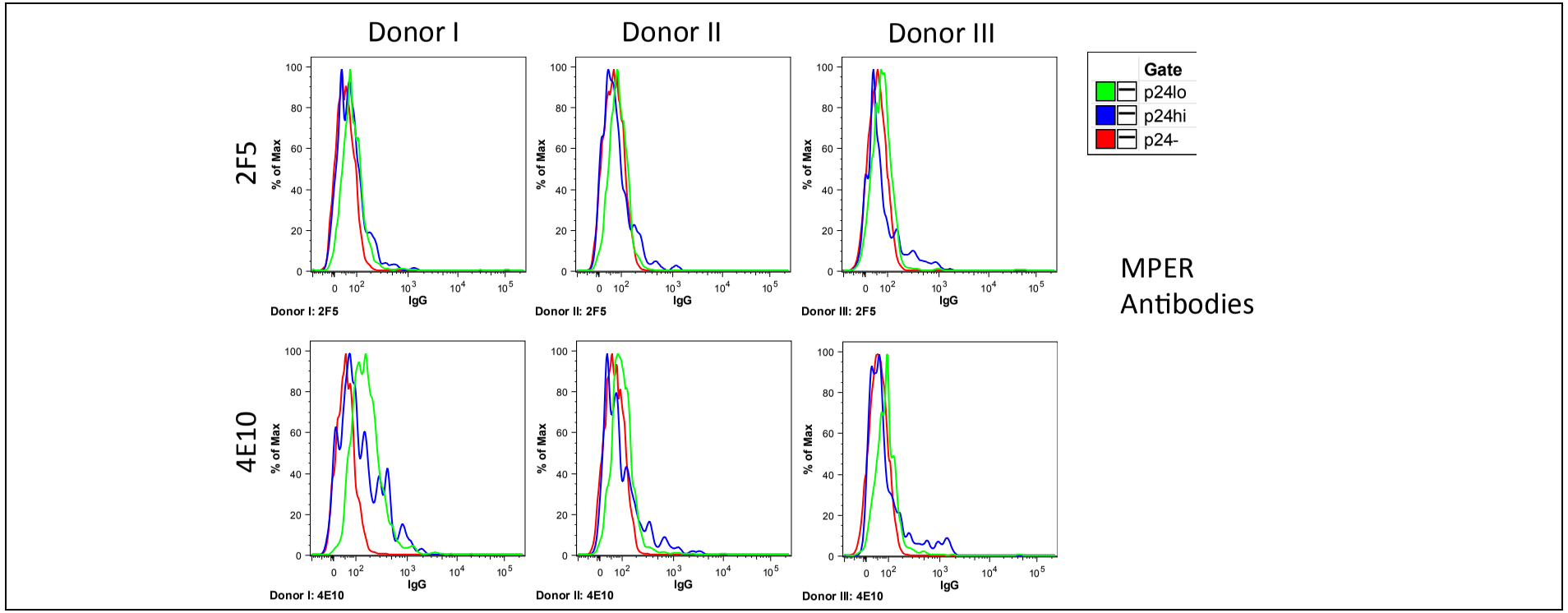


Figure 5.10 MPER-specific antibody binding

Histograms indicating MFI of 5 μg/mL IgG-specific binding to $CD4^{hi}p24^{lo}$ (green), $CD4^{lo}p24^{hi}$ (blue) and CD4+ p24- (red) cells infected with $HIV\text{-}1_{BaL}$ for 7 days.

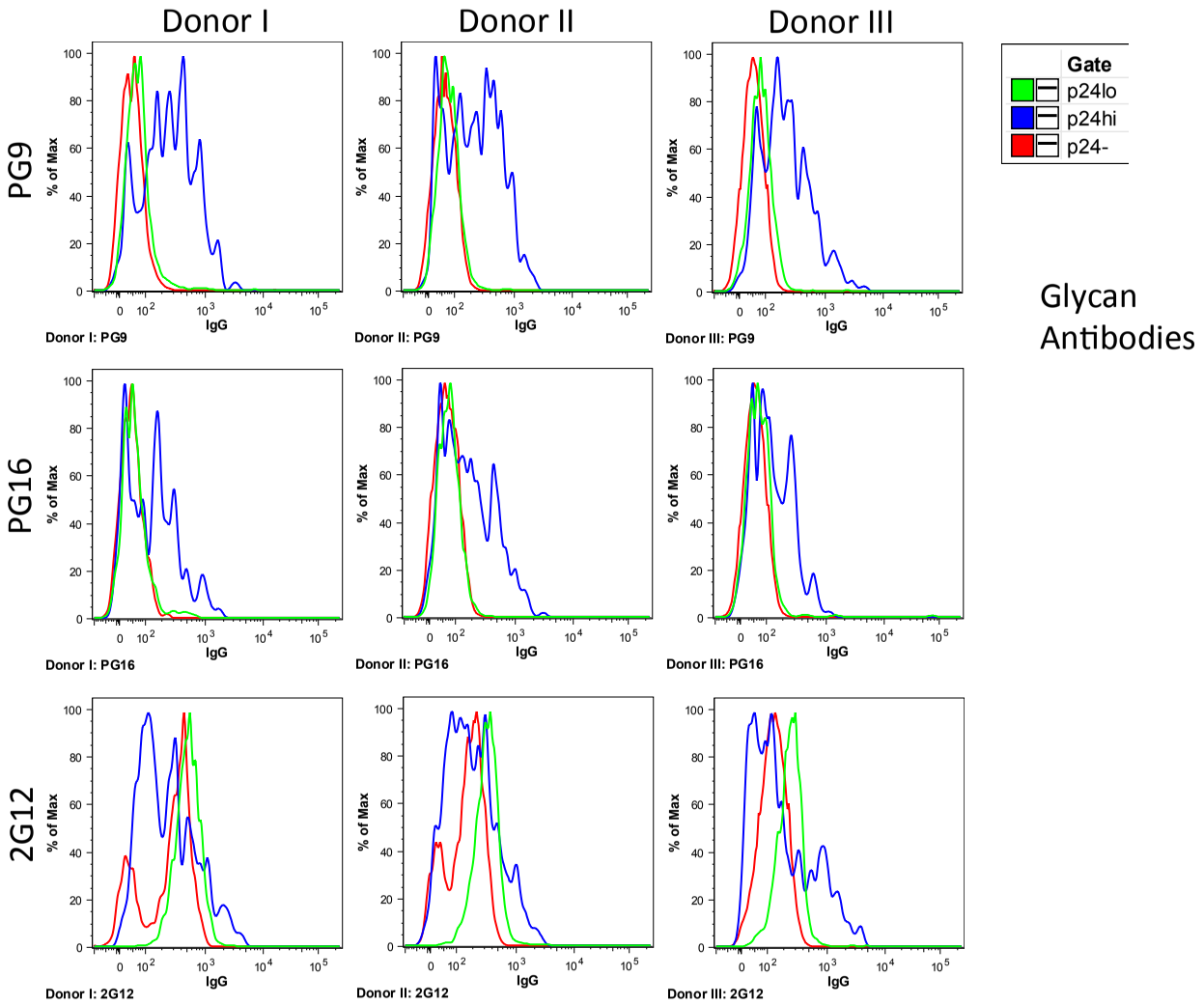


Figure 5.11 Glycan-specific antibody binding

Histograms indicating MFI of 5 μg/mL IgG-specific binding to $CD4^{hi}p24^{lo}$ (green), $CD4^{lo}p24^{hi}$ (blue) and CD4+ p24- (red) cells infected with HIV-1_{BaL} for 7 days.

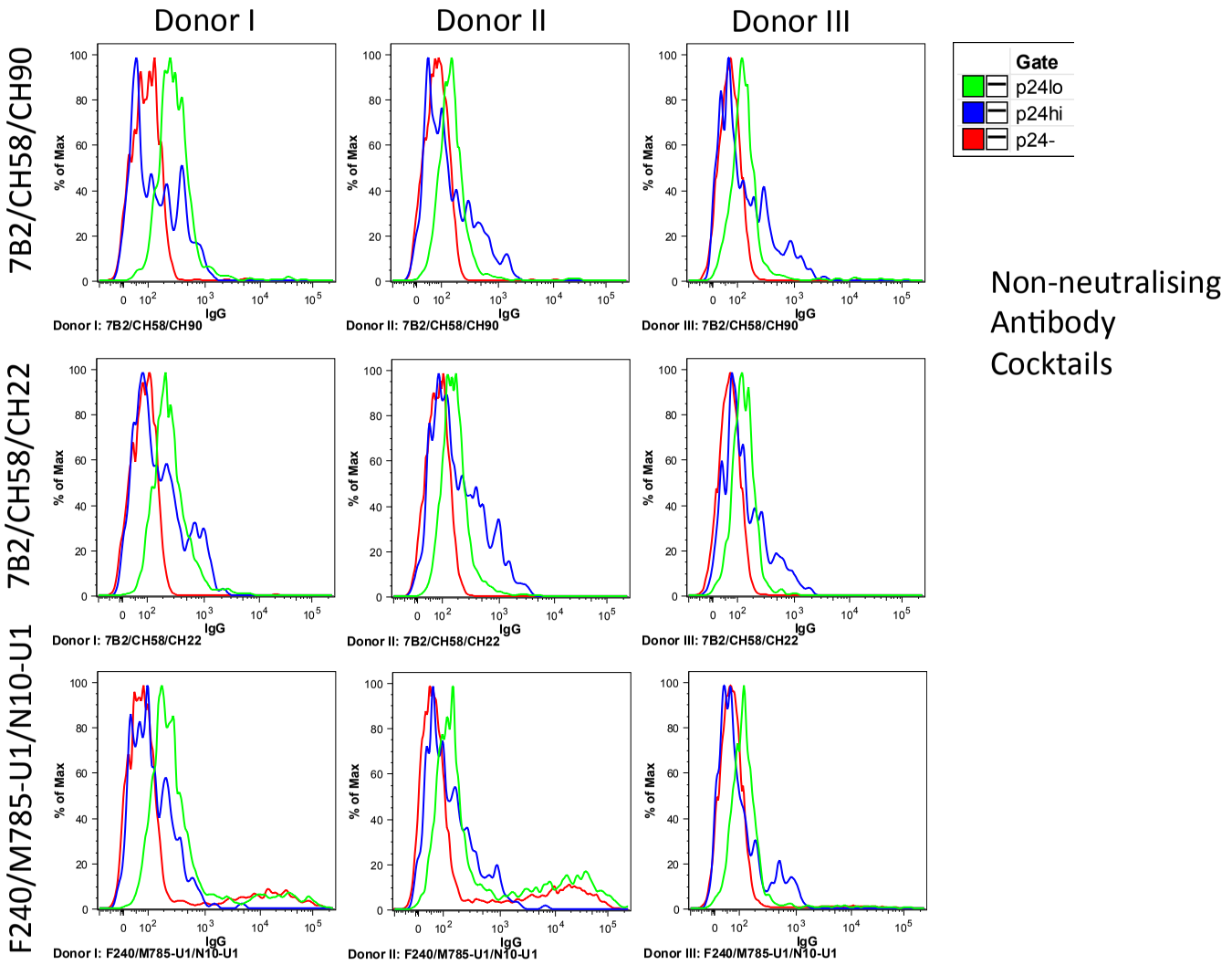


Figure 5.12 Non-neutralising antibody cocktail binding

Histograms indicating MFI of 5 μg/mL IgG-specific binding to $CD4^{hi}p24^{lo}$ (green), $CD4^{lo}p24^{hi}$ (blue) and CD4+ p24- (red) cells infected with HIV-1_{BaL} for 7 days.

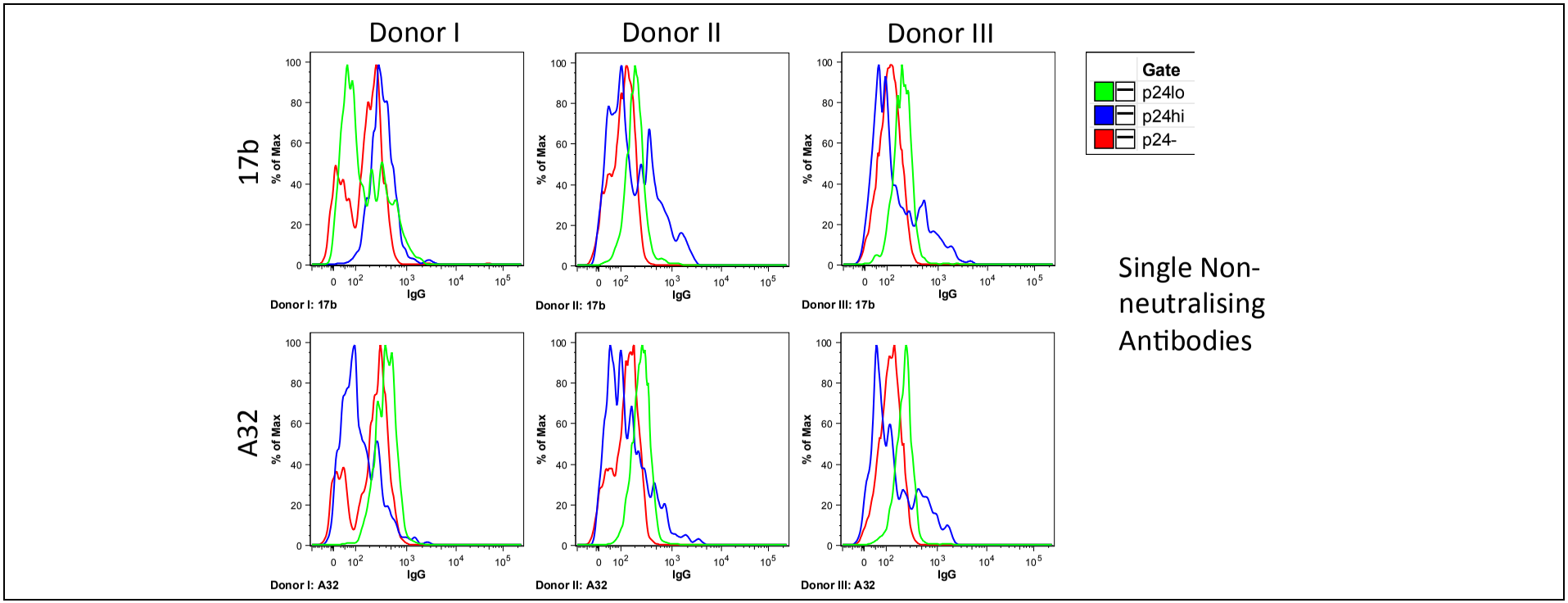


Figure 5.13 Single Non-neutralising antibody binding

Histograms indicating MFI of 5 μg/mL IgG-specific binding to $CD4^{hi}p24^{lo}$ (green), $CD4^{lo}p24^{hi}$ (blue) and CD4+ p24- (red) cells infected with HIV-1_{BaL} for 7 days.

5.2.5. Ability to Reduce Onward Transmission of HIV-1

The most efficient form of HIV-1 transmission *in vitro* is via cell-to-cell contact [251, 252]. The addition of cell trace violet to the whole blood assay system allowed for further investigation into the ability of the antibodies to prevent the onward transmission of HIV-1 to the CTV-CD4+ cells found within the whole blood added 24 hours before analysis. Percentages of p24+ cells were recorded in CTV-CD4+ cells and compared to the infected control. Further comparisons were then made between reduction of p24+ cells observed in CTV- and CTV+ CD4+ cells.

CD4bs-specific antibodies VRC01, b12 and CH31 significantly reduced CTV- p24+ cells by 84.7% [***P<0.001], 89.7% [***P<0.001] and 93.7% [****P<0.0001], respectively (Figure 5.14A). Although there was no significant difference observed between the CTV+ and CTV-CD4+p24+ cell percentages, there was a trend for greater reduction in the CTV-CD4+p24+ population for all the CD4bs-specific antibodies (Figure 5.14B).

MPER-specific 4E10 led to a significant reduction in the percentage of p24+ CTV- cells of 67.2% [**P<0.01] (Figure 5.14A). Although this was not as pronounced as the reduction of p24+ cells observed with CD4bs-specific antibodies, p24+ cell reduction induced by 4E10 in the CTV+ and CTV-CD4+p24+ cells were significantly different [*P<0.05] (Figure 5.14B).

For V1V2-specific antibody PG9, a significant reduction in the CTV- p24+ cell population was observed, 98.2% [****P<0.0001], whilst V3-specific 2G12, demonstrated a reduction in CTV- p24+ cells of 56.9% [*P<0.05] (Figure 5.14A).

When comparing CTV+ and CTV- populations, this difference only reached significance for PG9 [*P<0.05] (Figure 5.14B).

All of the nnAb cocktails significantly reduced the percentage of CTV-p24+ cells by approximately 75% [**P<0.01] (Figure 5.14A). When comparing the reduction of p24+ cells in the CTV+ or CTV- cell populations this difference only reached significance for 7B2/CH58/CH22 [***P<0.001] (Figure 5.14B). Furthermore, the single nnAb were not capable of reducing p24+ cells in either CTV+ or CTV- populations (Figure 5.14).

The reduction in CTV-p24+ cells (Figure 5.14A) are reflective of the inhibition observed in PBMC, as reported in Chapter 4 (Figure 4.8D) and potentially indicate a functional role in blocking HIV-1 transmission for the antibodies.

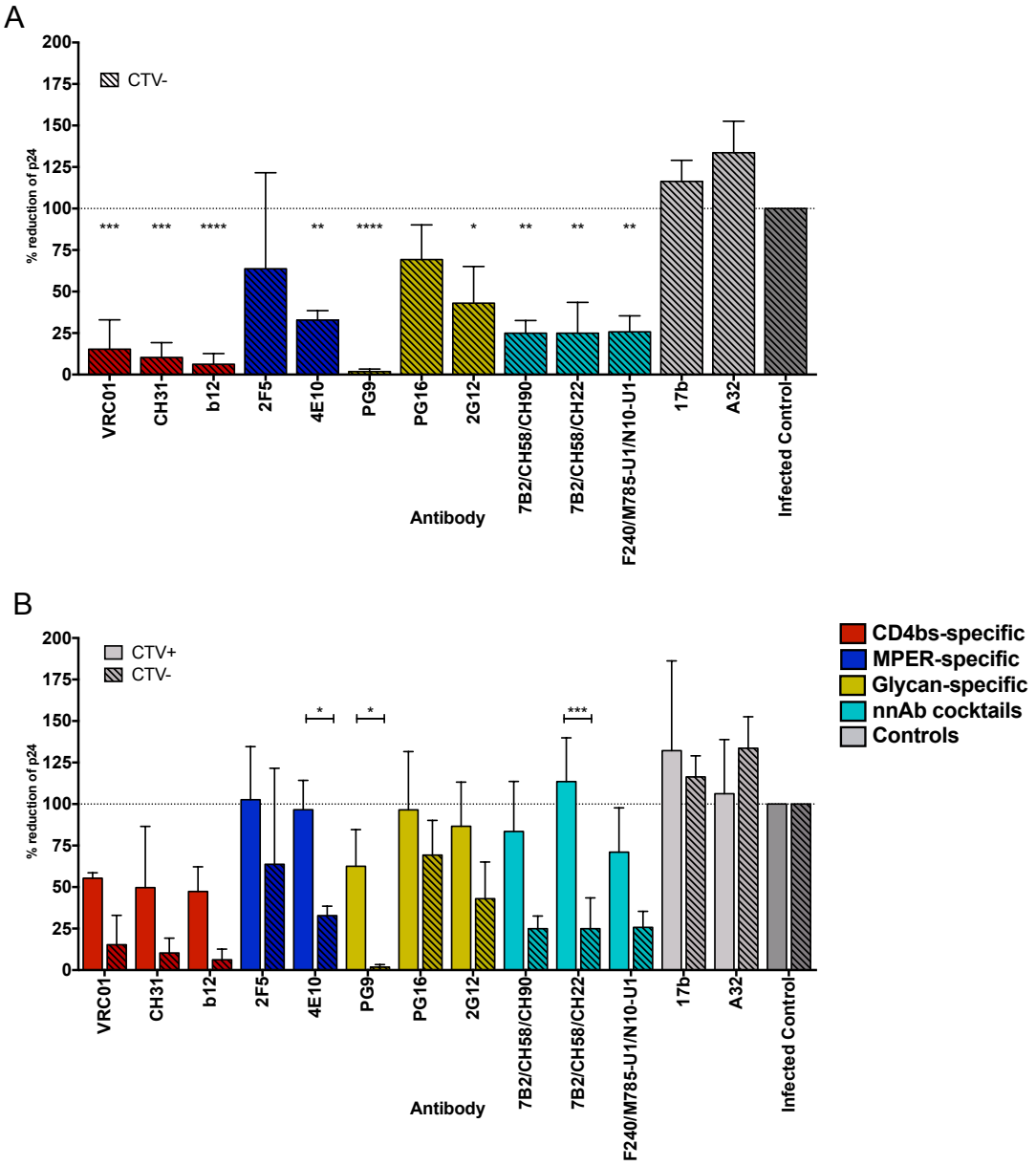


Figure 5.14 Reduction of p24+ cells in the CTV-CD4+ cell population after 24 h incubation versus that observed in the CTV+CD4+ population

The ability of nAb and nnAb (50 μg/mL or 25 μg/mL, each (cocktails)) to reduce percentage of p24+ cells with the addition of whole blood in (A) CD4+CTV- whole blood cells, incubated with the CTV+ cells for 24 hours (n=3) and (B) a comparison of the p24+ cell reduction observed between the CTV+ and CTV- CD4+ cells. Graphs show percentage p24 reduction in relation to the infected, media-only control. (A) statistical analysis performed using two-way ANOVA with Dunnett's multiple comparison correction. (B) statistical analysis performed using multiple t-tests with Holm-Sidak multiple comparison test [*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001].

5.3. Discussion

This chapter set out to investigate the functional activity of nAb and nnAb with or without the addition of whole blood to the assay system. This was done to assess the potential antiviral antibody effector functions that might be conferred by infiltrating peripheral immune cells in an attempt to further understand their potential for limiting HIV-1 infection at mucosal sites. Furthermore, the binding of HIV-1 antibodies to CD4+ cells was explored to establish a potential correlation between binding and reduction in p24+ cells.

The initial experiment to determine whether whole blood added to the assay system improved the activity of HIV-1 antibodies demonstrated significant reduction in the percentage of p24+ cells for all of the CD4bs nAb and for glycan-specific PG16 and PG9. However, the remaining nAb and nnAb tested did not reduce the percentage of p24+ cells by a significant amount. With the addition of whole blood, the percentage reduction of p24+ cells by the CD4bs nAb remained comparable to that observed without whole blood, potentially suggesting the addition of whole blood did not enhance the antiviral activity of CD4bs nAb. Conversely, the addition of whole blood to the assay system containing the nnAb cocktails demonstrated significant reduction in percentage of p24+ cells for all three cocktails (Figure 5.4). However, the assay system in this format was unable to discriminate between activity against the originally infected CD4+ cells from those introduced with the addition of whole blood after 7 days of infection. The use of cell trace violet dye enabled distinction between the two populations to be made. Similar to the results observed for the assay without the addition of whole blood, the CD4bs nAb all reduced the percentage of p24+ cells by approximately 50%, although significance was only reached for b12. The remaining antibodies

tested showed no ability to reduce the percentage of CTV+p24+ cells within the assay, indicating that the addition of whole blood did not enhance the activity of any of the antibodies or improve reduction of p24+ cells in already infected CD4+ cells (Figure 5.6). While the mechanism remains unknown, the reduction in HIV-1 infected cells by the CD4bs antibodies is reflective of a recent, small *in vivo* trial whereby VRC01 was given intravenously to HIV-1 positive ART-treated or untreated individuals. VRC01 showed a reduction in viral load of between 1.1 to 1.8 $\log_{10}$ in 6/8 ART-untreated viraemic subjects. Interestingly, the 2/8 ART-untreated subjects that did not respond to VRC01 infusion were subsequently found to be infected with VRC01-resistant virus [486]. This study highlights the potential for VRC01 as a therapeutic treatment for HIV-1, although the use of a single monoclonal antibody may not be enough to confer universal therapeutic activity.

Within this assay setting, it was not possible to determine if the reduction in p24+ cells was linked to ADCC (or other antibody-mediated functions). However, it would be of interest in future experiments to further explore these elements and also to determine whether pooling nAb into cocktails could boost the observed activity. Some caveats should be mentioned with regards to this assay. Reduction of p24+ cells was assessed by investigating percentage p24+ cells within the total viable cell population and percentage reduction was determined by comparing this value to that seen within the infected control. While this gives a good indication of whether an antibody can reduce the overall percentage of p24+ cells it cannot accurately determine depletion of CD4+ cell numbers. An ideal method would be able to assess whether the CD4+p24+ cells are being lost through antibody-mediated functions using flow cytometric bead

quantification during acquisition to normalise the data. Furthermore, although the data cannot determine whether the reduction in p24+ cells observed were associated with antibody- or complement-mediated functions such as ADCC, ADCP, ADCVI or CDC, it is possible that one of these mechanisms are responsible. Interestingly, others have demonstrated that nAb and sera from HIV-1 infected individuals are capable of exerting ADCC *in vitro* [483, 487]. However, these groups purified NK cell populations to assess the ADCC activity producing an artificial effector to target cell ratio not seen *in vivo*. By investigating cytokine expression and release within the assay system, it may be possible to establish a correlation with antibody activity and HIV-1 control. For example, a previous study investigating cytokine release in response to monoclonal antibody cancer therapy observed a positive correlation between ADCC and the release of IFN-γ, TNF-α, monocyte-chemotactic protein (MCP)-1 and IL-6. Interestingly, the authors also observed a negative correlation between release of the same cytokines and complement-dependent cytotoxicity [488]. Cytokine release assays have already been established for use in monoclonal antibody therapies for diseases such as cancer and could easily be modified and implemented here [489]. Furthermore, sequential deletion of individual cell subsets could allow further insight into the effector cell subset associated with the greatest viral control. By sequentially deleting individual cell subsets using methods such as magnetic beads, and examining the reduction of p24+ cells, combined with potential changes in the cytokine milieu, it might be possible to correlate antibody effector function with the antiviral activity observed.

During *in vivo* infection, peripheral cells are recruited to mucosal sites of HIV-1 infection by cytokines and chemokines [480, 481]. Furthermore, changes

in cytokine environment have been shown to alter the maturation status and FcR expression levels of a number of effector cells [153, 413, 415, 416, 490]. However, within this assay cells from whole blood were added without additional cytokines or chemokines. It would be relevant to investigate whether altering the cytokine environment in conjunction with the addition of whole blood influences the Fc-mediated activity of effector cells. Regardless of the mechanism, once fully optimised, this assay has the potential for use within vaccine clinical trials as a determinant of correlates of protection.

The results of the antibody binding experiments were interesting in that there was no apparent correlation between total binding observed and percentage p24+ cells. Preferential binding to the productively infected $CD4^{lo}p24^{hi}$ cell subset was observed for nAb VRC01, CH31, b12, 2F5, PG9 and PG16. Interestingly, the CD4bs-specific antibodies, which have consistently demonstrated the greatest ability to reduce HIV-1 infection in assays used throughout this thesis, were found within this group and demonstrated negligible binding to the $CD4^{hi}p24^{lo}$ or CD4+p24- cell subsets. Other groups have demonstrated a positive correlation between polyvalent sera antibody binding to infected cells and ADCC activity, although no association was observed between monoclonal antibody binding and ADCC [487]. However, it has been observed that efficient ADCC requires that there is sustained binding of nAb to an infected cell [483]. Furthermore, although this experiment could not identify binding of antibody to cell-free virus, other groups have shown that the most effective nAb are better at capturing free-virus [491]. Therefore, the improved antibody activity and lower cellular binding observed for CD4bs-specific

antibodies could be attributed to enhanced capture of free-virus over antibodies with different antibody specificities or functions.

2G12 was the only nAb where high binding to $CD4^{hi}p24^{lo}$ and CD4+p24- cell subsets was observed (Figure 5.8). Unfortunately, this assay design omitted key controls to establish non-specific background binding in a virus-free environment. This experimental control would be of particular relevance for deciphering the antibody binding patterns observed for 2G12 where the average MFI of 2G12 binding to $CD4^{hi}p24^{lo}$ and CD4+p24- cell subsets are higher when compared to the $CD4^{lo}p24^{hi}$ cell subsets (Figure 5.11).

The nnAb, both as cocktails and single antibodies, showed a tendency to bind to the $CD4^{hi}p24^{lo}$ or CD4+p24- cell subsets. This is in accordance with previous studies that have demonstrated non-neutralising HIV-1 epitopes found on the viral envelope structure are only exposed once the virus has bound to the CD4 entry receptor [34, 444, 492, 493]. Additionally, the apparent lack of binding by nnAb to infected cells could be also attributed to the induced downregulation of the CD4 receptor by HIV-1 viral protein, Vpu. Downregulation of the CD4 receptor prevents newly formed HIV-1 Env from binding to CD4 within the endoplasmic reticulum and therefore enhances virion assembly [28, 29]. However, this downregulation also reduces the ability of HIV-1 to bind to CD4+ T cells and, given that nnAb often require this interaction to expose binding epitopes, it also reduces the ability of nnAb to bind to infected cells. Furthermore, nnAb have been shown to preferentially bind to shed gp120, which could explain the binding observed on the CD4+p24- cell subset [243, 494]. NnAb interaction with $CD4^{hi}p24^{lo}$ or CD4+p24- cells could be of relevance mechanistically. Others have shown that binding of nnAb to non-infected CD4+ cells that have captured

virus or shed gp120 are subject to bystander killing through ADCC, exacerbating the CD4+ T cell loss seen during HIV-1 infection [243, 483]. Indeed, experiments using small CD4+ mimetics, compounds that bind to the CD4bs on cell-free virus or shed gp120, have shown some promising results *in vitro*. Introduction of the compounds into the assay system blocked binding of ADCC-inducing antibodies to bystander CD4+ T cells and also exposed HIV-1 infected cells to killing by ADCC [243]. It would be of interest to use such compounds within the experiments performed here to potentially enhance the activity of nnAb.

By examining the p24+ cells within the CTV-CD4+ cell fraction, it was possible to determine the impact of antibodies on reducing the onward transmission of HIV-1 during the 24 h incubation period post addition of whole blood. Both nAb and nnAb cocktails showed some promise at reducing the onward transmission of HIV-1. All CD4bs and nnAb cocktails, plus MPER-specific 4E10 and glycan-specific PG9 and 2G12 significantly reduced the percentage of p24+CD4+ cells found within the CTV- cell population (Figure 5.14).

Interestingly, several studies have indicated that some bnAb are less efficient at preventing cell-to-cell transfer of HIV-1 when compared to transmission by cell-free virus [253, 495]. However, it should be observed that both of these studies were performed in an environment lacking FcR-associated effector cells or complement. Furthermore, it cannot be excluded that the complement system may have an impact on HIV-1 transmission. Viral infection leads to an immediate response through the alternative complement pathway, while HIV-1 antibodies can further activate the classical complement pathway. The role of complement in HIV-1 infection remains controversial and largely understudied. Very early studies showed that complement enhanced HIV-1

infection [496, 497], while more recent work has indicated that it may also have a protective component [125, 498, 499]. However, previous work demonstrated that blocking CR3, but not FcR, reduced the killing of *Streptococcus pyogenes* by neutrophils [500]. Furthermore, a role for complement killing has been observed for Dengue virus, West Nile virus and Influenza [501-503]. However, NHP experiments using SHIV demonstrated that modifying the Fc-portion of nAb to reduce interaction with complement receptors did not adversely affect the neutralising capability of CD4bs antibody, b12 [385]. Despite this, it would be of interest to explore the function of complement within this assay system further. Receptor-blocking methods or the use of complement-blocking drugs [504] or modified antibodies could be implemented to determine the activity of the complement system at blocking HIV-1 cell-to-cell spread.

While HIV-1 infection of CD4+ cells occurs more readily if the cells are activated, the CD4+ cells added within the whole blood were not activated, making them less permissive to viral infection [505, 506]. However, studies have shown that resting CD4+ cells can become infected with HIV-1 [42, 43]. Results from Chapter 4 described the activity some of the antibodies used in this chapter in a series of cellular and mucosal tissue explant assays designed to mimic the early stages of infection. While only CD4bs antibodies consistently demonstrated an ability to inhibit HIV-1 infection across all models tested, some parallels can be drawn between the results seen in Chapter 4 and the results observed within this chapter. Although the nnAb were not capable of preventing onward infection of HIV-1 in the DC-T, tissue explant or migratory cell models, they efficiently inhibited infection within the macrophage, DC and PBMC models. Interestingly, these models were shown to contain high levels of FcR expressing cells. It is

therefore possible that the reduction in onward transmission of HIV-1 observed here is linked to Fc-mediated activity. The use of F'ab, F'ab2 or Fc/FcR-blocking could be employed in future experiments to confirm whether the antiviral activity observed here is linked to Fc-mediated effector functions. Indeed, previous NHP studies have demonstrated a reduction in the activity of nAb against SIV/SHIV infection when the Fc-region is removed or mutated [385, 455].

Overall, this chapter sought to develop a whole blood assay to determine whether infiltrating cells from the periphery might have the potential to enhance antiviral antibody activity during the early stages of HIV-1 infection at mucosal sites. Furthermore, this chapter sought to characterise the binding abilities of these antibodies and to correlate binding with antibody activity. To summarise, the data indicate that:

- CD4bs-specific nAb consistently demonstrated the greatest reduction of p24+ CD4+ cells, irrespective of the addition of whole blood.
- MPER- and glycan-specific nAb and nnAb (both in cocktail or as single antibodies) were not able to reduce the percentage of p24+CD4+ cells, irrespective of the addition of whole blood.
- Three antibody binding profiles were observed; a) preferential binding to $CD4^{lo}p24^{hi}$; b) preferential binding to $CD4^{hi}p24^{lo}$ and c) preferential binding to CD4+p24-. The overall amount of binding observed did not correlate with reduction in p24+ cells.

- With the exception of the single nnAb, MPER-specific 2F5 and Glycan-specific PG16, all antibodies tested reduced the onward transmission of HIV-1_{BaL}.

Although there are several ways by which this assay design could be improved, the results demonstrate that the addition of whole blood does not appear to enhance the activity of any of the antibodies at reducing the percentage of HIV-1 infected CD4+ cells. This suggests that infiltrating cells from the periphery may be insufficient to eradicate the primary foci of infection within mucosal tissues, further investigations are warranted to determine if this is indeed the case. However, the majority of antibodies were able to reduce the onward transmission of HIV-1, which may be relevant during the early stages of infection. Additionally, this chapter supports the experimental work described in Chapter 4 and further defines the potential for CD4bs antibodies as promising therapeutic agents for the prevention of HIV-1 infection.

CHAPTER 6 – DISCUSSION AND CONCLUSIONS

6 Discussion and Conclusions

HIV-1 has been a global epidemic for more than thirty years. In 2015, 36.7 million people were thought to be living with HIV-1, with around 17 million having access to antiretroviral therapy (ART) [www.unaids.org]. Despite advances in ART, a preventative vaccine is still thought necessary to further slow the epidemic, particularly in developing nations, where access to ART is more limited. However, many challenges remain for the development of an HIV-1 vaccine. The RV144 trial suggested that nnAb and antibody-dependent effector functions could be important. NnAb are more readily induced post-infection, although they are to all forms of the envelope spike, both functional and non-functional [34]. Furthermore, difficulties exist in the induction of bnAb, such as clonal deletion, immune tolerance, the need for high levels of somatic hypermutation and long HCDR3 regions; all of which will need to be overcome [35, 220-222, 442]. However, even if bnAb are induced, it has been shown that Fc-mediated effector functions enhance their activity *in vivo* [386, 483]. With the majority of HIV-1 transmission events occurring at mucosal surfaces via sexual transmission, the ability of immune cells within these tissues to mount an effective immune response is important to define.

Significantly, this thesis investigated the expression profile and location of FcR, CD16 (FcγRIII), CD32 (FcγRII), CD64 (FcγRI) and CD89 (FcαR) within colorectal, ectocervical and penile glans tissue, as well as defining the effector cell populations found within these tissues. This is the first time this has been described for all tissues associated with mucosal transmission of HIV-1. Furthermore, the finding that nnAb are insufficient to prevent HIV-1 infection of

the three different mucosal tissues associated with HIV-1 transmission has not been shown before. Finally, the development of an assay to determine whether infiltrating cells from the periphery have the potential to enhance Fc-mediated response has not been previously investigated.

6.1 Conclusions

The initial aim of this thesis was to characterise the expression profiles of FcR in mucosal tissue associated with HIV-1 transmission, specifically colorectal, penile glans and ectocervical. This was important to define from the outset, as lack of FcR or associated effector cells with mucosal tissue would potentially limit the activity of nnAb at sites of HIV-1 transmission. The data revealed:

- Significantly lower levels of FcR expression on effector cells isolated from the mucosal tissues when compared to whole blood.
- FcR expressing myeloid cells isolated from mucosal tissue had higher variation in FcR expression than cells from the periphery, although the majority of cells isolated from mucosal tissues were completely negative for any FcR expression.
- Cells isolated from whole blood were predominantly FcR-expressing.
- FcR-expressing cells were located deep within tissue and therefore not readily accessible to mucosally-induced antibody.
- There may be insufficient FcR expression within mucosal tissue to respond to nnAb in an Fc-mediated manner.

Sips *et al*. recently demonstrated similar FcR expression in cervical and colorectal tissue, although this study failed to report the FcR-negative fraction or compare the FcR expression to that seen in peripheral cells [174]. While comment cannot be made on the functional activity of these mucosally-derived FcR expressing effector cells, the research undertaken in this thesis is the first time FcR expression has been investigated across multiple mucosal tissues, including penile glans.

Having defined FcR expression within mucosal tissue, the second aim of the thesis was to determine the antiviral activity of nAb and nnAb in different cellular and mucosal tissue models designed to mimic the early stages of infection. The results revealed that:

- FcR expression on PBMC, macrophages and dendritic cells used within the cellular assays was high and good levels of antiviral activity were observed within these assays, irrespective of conventional antibody neutralisation.
- Single nnAb showed limited antiviral activity within the cellular models and were pooled into cocktails in an attempt to enhance the inhibitory activity within mucosal tissue. However, while these cocktails demonstrated high levels of inhibitory activity within the majority of cellular models, their activity within the mucosal tissue models was limited.
- Across all cellular and tissue models, CD4bs-specific nAb consistently displayed the greatest ability to inhibit HIV-1 infection,

while MPER- and glycan-specific nAb only demonstrated efficient inhibition in the cellular models.

- In a static system, nnAb were insufficient to prevent HIV-1 infection, whereas CD4bs-specific nAb displayed superior ability to inhibit infection irrespective of the cellular or tissue model.
- While other groups have investigated the activity of nAb and nnAb within tissue explant models, this is the first study to investigate antiviral antibody activity across colorectal, ectocervical and penile glans tissue.

Taken together the data from the first results chapters indicated that mucosal tissue in isolation could not mount a sufficient antibody mediated effector immune response to inhibit HIV-1 infection. Thus, the final aim was to determine whether infiltrating immune cells from whole blood could enhance antiviral antibody activity to HIV-1. Furthermore, the binding of HIV-1 antibodies to infected cells was investigated to attempt to correlate binding with antiviral function. The results suggest that:

- CD4bs-specific antibodies were the most effective at reducing HIV-1 infection, irrespective of the addition of whole blood.
- Conversely, the remaining nAb and nnAb tested demonstrated no ability to reduce HIV-1 infection, irrespective of the addition of whole blood.

- With the exception of MPER-specific 2F5 and glycan-specific PG16, all antibodies investigated reduced the onward transmission of HIV-1.
- The binding of antibody to CD4+ cells within the assay revealed three antibody binding profiles; a) preferential binding to $CD4^{lo}p24^{hi}$; b) preferential binding to $CD4^{hi}p24^{lo}$ and c) preferential binding to CD4+p24-.
- These profiles did not correlate with reduction in HIV-1 infection.

We believe that this is the first time an *in vitro* whole blood assay has been used to assess the antiviral activity of HIV-1 specific antibodies. Overall, the results demonstrate that infiltrating cells from the periphery may be insufficient to eliminate already infected cells, but they may have a function in preventing onward cell-to-cell transmission in the presence of HIV-1 antibodies.

6.2 Limitations and Future Work

One limitation of this study is that $HIV\text{-}1_{BaL}$ is the only virus used throughout. $HIV\text{-}1_{BaL}$ is a clade B macrophage-tropic tier 1 virus, chosen for use in these studies as it readily infects CD4+ T cells and macrophages within tissue. In addition, it is more easily neutralised when compared to a tier 2 virus or some transmitter/founder strains. These two character traits were integral in the rationale behind choosing $HIV\text{-}1_{BaL}$ for use in the experiments throughout this thesis. However, it would be relevant in the future to investigate the antiviral

activity of antibodies against other viral strains and clades to determine whether similar inhibition of HIV-1 is observed.

Another limitation may be that all antibodies used throughout this thesis were of the IgG1 subclass. Differences between IgG subclasses in their ability to induce Fc-mediated effector functions are known and it would therefore be important to further examine the role of these different subclasses at inhibiting HIV-1 infection, both for nAb and nnAb. Furthermore, IgA is the most prevalent immunoglobulin at mucosal surfaces, so it would be of interest to further examine the ability of IgA1 and IgA2, in both monomeric and dimeric forms, to inhibit HIV-1 infection within mucosal tissues. Moreover, future experiments could investigate F'ab and F'ab2 fragments or Fc/FcR-blocking to determine whether the antiviral activity is linked to Fc-mediated effector functions. Lastly, it would be relevant to investigate pooling nAb in an attempt to further enhance the inhibitory effect observed for these antibodies.

While the main objectives of this thesis were to investigate antibody-mediated effector functions, future work should be conducted to gather more information as to the role of antiviral activity conferred by complement within these mucosal tissues and assay systems. This could be achieved using complement receptor-blocking methods, the use of complement-blocking drugs [504] or Fc-mutated antibodies that lack the ability to bind complement.

Throughout this thesis, tissue samples were acquired from patients attending clinics in London, UK. While information regarding the demographics of these patients was not obtained, it is probable that these individuals were not living outside the UK. Of relevance here could be external influences that can alter FcR expression, with environmental factors such as *Plasmodium falciparum*

infection, diet and the use of pro- and antibiotics shown to have an effect [417, 418, 421, 422]. Therefore, it would be of great interest to determine the FcR expression and perform the tissue explant experiments using tissue obtained from a developing nation. In addition, at risk patients have a greater probability of being co-infected with other STIs, which may also alter the FcR expression and inflammatory immune environment. It may be important to further define the impact of concurrent STI infections on the activity of HIV-1 antibodies at sites of mucosal transmission. Furthermore, as it is known that cyclical hormonal fluctuations can alter the cytokine environment within the female reproductive tract [266, 267], it would be key to determine the FcR expression within cervical tissue at different stages of the menstrual cycle.

When characterising FcR expression for CD16 and CD32, due to antibody availability, antibodies were used that could not distinguish between the inhibitory and activating forms of these receptors. Given the varied functions of the receptors in terms of inducing either immune tolerance (inhibitory) or downstream antibody-mediated effector functions (activating), such as ADCC, ADCP and ADCVI, it would be of interest to determine the relative expression of these receptors within tissue. Similarly, it was not possible to investigate allelic variation between donors, which could be applicable when investigating Fc-mediated antibody activity in larger studies with donors from wider geographical areas.

In future experiments, it would be of relevance to determine the role of cytokines and chemokines on FcR expression within tissue and on the impact of introducing these components into the tissue or whole blood assay systems to potentially increase the activity of effector cells. Furthermore, the use of small

CD4+ mimetics to possibly enhance the activity of nnAb warrants further investigation.

The whole blood assay system requires some additional optimisation to enable it to deliver a more complete set of results. By measuring cytokine release, it might be possible to establish a correlation with antibody activity and HIV-1 control. This could be achieved by employing techniques such as luminex, cytometric bead array or ELISA to determine the cytokine expression profiles and relate them back to function. Furthermore, it is not possible to quantify the loss of CD4+ T cells from the system through antibody-mediated activity, such as ADCC, ADCP and ADCVI. Introducing flow cytometric beads into the assay would enable quantification of the data. Similarly, when characterising the effector cell profiles within mucosal tissues, it was not possible to enumerate the cell numbers. The use of flow cytometric beads has the potential to better quantify the cells isolated from mucosal tissue.

Short-term future goals would include performing the experiments with multiple viral strains and clades and different antibody isotypes and subclasses, as well as investigating the role of cytokines and small CD4 mimetic compounds at enhancing the activity of nnAb. In the long-term, performing the experiments with tissue samples from donors from developing nations and from those at high-risk of infection to understand the impact of environmental influences on FcR expression and subsequent antibody activity within the tissue models would be of relevance.

6.3 Future for HIV-1 Vaccine Design

Despite the challenges, it is now widely accepted that a successful HIV-1 vaccine must induce a broad, multi-faceted immune response. However, a better understanding of the correlates and mechanisms of protection for HIV-1 are needed, particularly at mucosal surfaces.

The use of novel viral vectors to increase the immunogenicity of vaccines has demonstrated potential in recent years. NHP studies using cytomegalovirus (CMV) vectors have shown that it is possible to induce broad central memory T cell immunity to SIV infection [507], although the use of CMV as a viral vector in human clinical trials remains controversial.

While the limited protection elicited by the RV144 vaccine trial correlated with nnAb and antibody-effector functions, the focus of HIV-1 vaccine design should not solely be on the induction nnAb. Recent mouse experiments have demonstrated that it is possible to guide the development of early germline B cells toward bnAb production [225-227]. Although it remains to be determined whether this can be achieved in humans, it has given fresh optimism to the vaccine field that the goal of inducing bnAb through vaccination can be reached. Moreover, the results from a recent study demonstrated that a new generation glycan-specific bnAb, PGT121, could partially block mucosal SHIV infection in NHP, although some viral escape to distal tissues was detected. Upon closer investigation, the authors observed progressive clearance of the virus from these tissues over a 7-day period, indicating that bnAb can exert antiviral activity even after the infection has spread to distal tissues [508]. The results suggest that a vaccine capable of eliciting bnAb responses may not have to block all viral

transmission at mucosal surfaces if they can exert activity once distal infection has been established.

6.4 Final Remarks

A prophylactic HIV-1 vaccine remains crucial for halting the HIV-1 epidemic. Successful vaccine-induced immunity will most like require both T- and B cell responses.

The primary aims of this thesis were to investigate the protective efficacy of nAb and nnAb at preventing HIV-1 transmission within mucosal tissues and to determine whether the functional activity was linked to conventional neutralisation or Fc-mediated effector functions. Work carried out in this thesis suggests that:

- FcR expression on cells isolated from mucosal tissues is lower than that found on cells isolate from the periphery, an important detail when attempting to engage Fc-mediated functions to enhance the activity of HIV-1 antibodies.
- nAb display superior abilities to prevent infection over nnAb.
- nAb hold the greatest potential for eliminating HIV-1 infected cells, while nnAb may have some impact at reducing onward transmission.

Overall, the results from this thesis suggest that nnAb and Fc-mediated effector functions may not be sufficient to block HIV-1 transmission at mucosal surfaces. Future vaccine design should focus on inducing a nAb response that will engage all elements of the innate and adaptive immune responses.

7 References

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