CELLULAR TROPISM OF HUMAN IMMUNODEFICIENCY VIRUS: RECEPTORS AND INHIBITORS

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A Thesis submitted to the University of London
for the degree of Doctor of Philosophy

June 2003

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HIGHLY ACTIVE ANTI-RETROVIRAL THERAPY (HAART) has been very effective in reducing viral loads in HIV+ patients. However, current therapies carry detrimental side effects, require complex drug regimes for administration and are threatened by the emergence of resistant variants. The need for new effective therapies targeted to different stages in the HIV life cycle is urgent. The 7-transmembrane G protein-coupled chemokine receptors CCR5 and CXCR4 are the major coreceptors for HIV and SIV. The majority of transmitted viruses are R5-tropic, yet variants able to exploit CXCR4 (R5X4- or X4-tropic) emerge in the late stages of disease in up to 50% of individuals. As R5 strains remain present throughout disease, CCR5 is an ideal target for novel therapeutic intervention. At least a dozen other chemokine receptors or close relatives also support infection by particular HIV/SIV strains on CD4+ indicator cell lines in vitro. Despite the expression of many of these different receptors on primary CD4+ cells, their role during in vivo infection is currently thought to be insignificant. However, in the advent of CCR5 inhibitors, minority populations of variants able to use such coreceptors may become predominant and thus escape inhibition by CCR5-specific drugs.

Here I have analysed the sensitivity of R5 and R5X4 strains of HIV and SIV to a series of six novel small molecule inhibitors of CCR5 on a diverse range of cell types, including lymphocytes and macrophages, the main cell types targeted by HIV-1 in vivo. In order to better evaluate the contribution of alternative coreceptors in vivo in the event of CCR5 being blocked, several primary untransformed cell cultures, including peripheral blood mononuclear cells (PBMCs), brain microvascular endothelial cells (BMVECs) and cells from immunoprivileged sites such as astrocytes and Leydig cells, were tested for expression of functional coreceptors able to support infection by HIV and SIV. A coreceptor of unknown identity has been discovered that is expressed on these primary cells and that supports infection by a subset of HIV and SIV isolates, including some from both subtypes B and C. The potential in vivo roles of CCR5 inhibitors and of alternative coreceptors will be discussed.
ACKNOWLEDGEMENTS

Firstly I thank my supervisor Paul Clapham. His patience, wealth of knowledge, and continued belief in me and this thesis have been invaluable. I am fortunate to have worked in two institutes during my PhD. The Wohl Virion Centre is a very special place. Robin Weiss’ zest for both life and science is unrivalled, and has created a unique and inspirational place to work. My gratitude goes to Áine McKnight, who took me under her wing in Paul’s absence, and read my thesis with such short notice! Many thanks to Graham for showing me the way in virology, and to all my friends and colleagues in the Wohl who made working there an absolute pleasure. Particular thanks to Elaine, Sam, Keith, and Cécile, and to Stuart for reading my thesis. At UMASS Medical School, I am indebted to Mario Stevenson for getting me interested in HIV, to the Stevenson lab for providing me with lab space when I first arrived, and the Sullivan/Luzuriaga labs for their generosity with time, reagents, equipment and expertise. Special thanks to Jean-Marc for many things - reading my papers, reading my thesis, and most importantly, the musical interludes. Final thanks to my friends in the States for making my final year such fun – Viv, Jaymes, Fee, Vicki, Catherine and Nancy.

This thesis was supported by Pfizer Global Research and Development, Sandwich. I thank Manos Perros, Pat Dorr, Graham Rickett and Chris Pickford at Pfizer GRD for reagents, ideas and support.

David, I can’t thank you enough. Thank you for helping me focus on the important things in life, for your continual support, for helping me print and for being there for me whilst I was away. I know it has been difficult and I just hope I can do the same for you. I love you with all my heart.

Lastly I thank my family. My sister Jo and brother Si have always believed in my work, and me, and this has lifted me up more times than I can count. Thank you both. Strongest thanks go to my parents, Ian and Brenda, whose enthusiasm and support for all I have done, and will go on to do, has been invaluable. Dad, your innate belief that I could do this has helped me beyond words. Mum, your love and pride in all your children will never be forgotten, and to you I dedicate my thesis.
For Mum
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ABBREVIATIONS

7TM        seven transmembrane
aa         amino acid
ADC        AIDS dementia complex
AIDS       acquired immunodeficiency syndrome
ALV        avian leukaemia virus
ARC        AIDS-related complex
BBB        blood:brain barrier
BLV        bovine leukaemia virus
BMVEC      brain microvascular endothelial cells
bp         base pair
dd         double distilled
CA         capsid protein
CAF        CD8+ T-cell antiviral factor
CCR        CC chemokine receptor
CHO        Chinese hamster ovary cells
CMV        cytomegalovirus
CNS        central nervous system
CRF        circulating recombinant form
CSF        cerebral spinal fluid
CTL        cytotoxic T lymphocyte
CXC        CXC chemokine receptor
DC         dendritic cell
DC-SIGN    DC-specific ICAM-3-grabbing non-integrin
DMEM       Dulbecco's modified Eagle medium
DMSO       dimethyl sulfoxide
DNA        deoxyribonucleic acid
dNTP        deoxynucleotide
EIAV       equine infectious anaemia virus
ELISA      enzyme-linked immunosorbent assay
env        viral envelope gene
ER         endoplasmic reticulum
ESN        exposed seronegative individual
Fab        antigen-binding fragment of antibody
FACS       fluorescence activated cell sorting
FCS        foetal calf serum
FDC        follicular dendritic cells
FeLV       feline leukaemia virus
FFU        focus-forming units
FITC       fluorescein isothyocyanate
FIV        feline immunodeficiency virus
FSC        forward scatter
g          g-force
Gag        group-specific antigen gene
GAGs       glycosaminoglycans
GalCer     galactosylceramide/galactocerebroside
GaLV       gibbon ape leukaemia virus
GFAP       glial acidic fibrillary acidic protein
GM-CSF     granulocyte/monocyte colony stimulating factor
GPCR       G-protein coupled receptor
gp41       transmembrane subunit of the HIV envelope
gp120      outer envelope subunit of the HIV envelope
HAD        HIV-associated dementia
HAG        heat aggregated γ-globulin
HBSS       Hanks buffered saline solution
HCC-1       hemofiltrate C-C chemokine 1
hEGF       human endothelial growth factor
hFGF       human fibroblast growth factor
HIV        human immunodeficiency virus
HIVE HIV-encephalitis
HFV/SFVcpz human foamy virus/simian foamy virus
HTLV-I/II human T-lymphotropic virus I/II
ICAM-3 intercellular adhesion molecule 3
IDC interdigitating dendritic cells
IGF insulin growth factor
IL-2 interleukin 2
IN integrase
IPTG isopropyl β-D-thiogalactoside
IVDU intravenous drug user
JSRV Jaagsiekte sheep retrovirus
kb kilobase
kDa kilodaltons
KSHV Kaposi's Sarcoma associated herpesvirus
LTNP lon-term non-progressor
LTR long terminal repeat
MA matrix
MAPK mitogen-activated protein kinase
MCP-1 monocyte chemotactic protein 1
MCP-2 monocyte chemotactic protein 2
MCP-3 monocyte chemotactic protein 3
MDC macrophage-derived chemokine
mDC myeloid dendritic cells
MIP-1 macrophage inflammatory protein 1
MNGC multi-nucleated giant cells
MPMV Mason-Pfizer monkey virus
mRNA messenger ribonucleic acid
MSX methionine sulfoximine
MuLV murine leukaemia virus
NC nucleocapsid
NNRTI non-nucleoside reverse transcriptase inhibitor
NRTI nucleoside reverse transcriptase inhibitor
ORF open reading frame
PBMCs peripheral blood mononuclear cells
pcDC plasmacytoid dendritic cells
PCR polymerase chain reaction
PE phycoerythrin
PHA phytohaemagglutinin
PI protease inhibitor
PIC pre-integration complex
Pol polymerase protein
PR protease protein
PTK phosphotyrosine kinase
R5 CCR5-tropic
RANTES regulated upon activation, normal T cell expressed and secreted
Rev regulator of viral protein expression
RNA ribonucleic acid
RPMI Roswell Park Memorial Institute
RRE Rev responsive element
RSV Rous sarcoma virus
RT reverse transcriptase
RTC reverse transcription complex
RT-PCR reverse transcriptase-polymerase chain reaction
sCD4 soluble CD4
SDF stromal derived factor
sGp120 soluble gp120
STD sexually transmitted disease
SIV simian immunodeficiency virus
SSC side scatter
STI structured treatment interruption
SU retroviral surface glycoprotein
TAR transactivation responsive element
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>TARC</td>
<td>thymus and activation-regulated chemokine</td>
</tr>
<tr>
<td>Tat</td>
<td>retroviral transactivator of transcription</td>
</tr>
<tr>
<td>TCLA</td>
<td>T-cell line adapted</td>
</tr>
<tr>
<td>TECK</td>
<td>thymus-expressed chemokine</td>
</tr>
<tr>
<td>TM</td>
<td>retroviral transmembrane glycoprotein</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumour necrosis factor α</td>
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<tr>
<td>vCKBP</td>
<td>viral chemokine binding protein</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial cell growth factor</td>
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<tr>
<td>Vif</td>
<td>viral infectivity factor</td>
</tr>
<tr>
<td>vMIP-I</td>
<td>viral macrophage inflammatory protein 1</td>
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<tr>
<td>vMIP-II</td>
<td>viral macrophage inflammatory protein 2</td>
</tr>
<tr>
<td>VMV</td>
<td>visna/maedi virus</td>
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<tr>
<td>Vpr</td>
<td>viral protein R</td>
</tr>
<tr>
<td>Vpu</td>
<td>viral protein U</td>
</tr>
<tr>
<td>Vpx</td>
<td>viral protein X</td>
</tr>
<tr>
<td>X4</td>
<td>CXCR4-tropic</td>
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CHAPTER 1

Introduction

1.1 Biology of HIV

1.1.1 Discovery of HIV

Acquired immunodeficiency syndrome (AIDS) was recognised as a disease in 1981 when groups of homosexual men in the USA, and later in Europe, presented with symptoms of a depressed immune system despite no previous history of immune defects (Masur et al., 1981; Mildvan et al., 1982; Siegal et al., 1981). Cases of the ‘gay compromise syndrome’ (Brennan and Durack, 1981) were also reported in intravenous drug users, individuals of Haitian origin, as well as blood transfusion patients and haemophiliacs (Dozier et al., 1983; Elliott et al., 1983; Khan and Wollschlorger, 1983; Poon et al., 1983; Shannon et al., 1983). In 1983 it was discovered that some infants and children born into families at risk from AIDS were suffering from the same immune disorders as those found in the gay community (Cowan et al., 1984; Elliott et al., 1983; Scott et al., 1984).

The epidemiology of this acquired immune dysfunction, which was transmitted sexually or via contaminated blood and blood products, as well as vertically from an infected mother to her child, indicated that an infectious agent was responsible for AIDS (Francis, Curran, and Essex, 1983). Indeed, three groups isolated and identified a novel virus belonging to the Retroviridae family from PBMC cultures from AIDS patients (Barre-Sinoussi et al., 1983; Gallo et al., 1984; Levy et al., 1984; Popovic et al., 1984). The infectious agent, lymphadenopathy associated virus (LAV), human T-cell leukaemia virus III (HTLV-III), or AIDS-associated retrovirus (ARV), was originally believed to belong to the family of human T-cell leukaemia viruses, as the target cell for this virus was the CD4^ T-cells. However, differences in virus ultrastructure and a lack of reactivity to HTLV-specific antibodies led to the realisation that a new human retrovirus had been found (Gallo, 2002; Montagnier, 2002; Montagnier et al., 1984). This virus was later named human immunodeficiency virus (HIV)(Coffin et al., 1986).

Around the same time that HIV was discovered, colonies of captive rhesus macaques were found to have AIDS-like symptoms, although this was later found to be
caused by simian retroviruses 1 and 2 (Henrickson et al., 1983; Letvin et al., 1983; Simon et al., 1998a). Subsequently, a T-cell tropic retrovirus, termed STLV-III was isolated (Daniel et al., 1985). When virus isolated from West African AIDS patients in 1986 was found to be closely related to STLV-I, yet distinct from LAV, the second human immunodeficiency virus, HIV-2 (previously LAV-2), was discovered (Clavel et al., 1986).

1.1.2 Taxonomy of HIV

HIV belongs to the lentivirus genus of the family Retroviridae. This class of RNA viruses are characterised by their life cycle, whereby the viral genome is reverse transcribed from single-stranded RNA to double-stranded DNA by the enzyme reverse transcriptase (RT), and integrated into the host DNA upon entry into the host cell (section 1.5)(Goff, 2001; Temin, 1992). All known retroviruses possess the same structural polyproteins. Gag encodes the structural proteins matrix (MA), capsid (CA) and nucleocapsid (NC), Pol encodes the three enzymes RT, integrase (IN) and protease (PR), and Env encodes the envelope proteins gp120 and gp41. As such, retroviruses share a common virus particle structure. The spherical shape of the outer lipid bilayer, taken from the host cell upon budding, is maintained by a layer of viral protein which in turn surrounds the central nucleocapsid core (Fig. 1.6). The morphology of the central nucleocapsid core, as seen by electron microscopy, differs between viruses and was originally used to classify retroviruses. For example, A-type retrovirus cores have a thick shell and hollow centre, B-types have a round off-centre core, and D-types have a cylindrical core. Retroviruses have also been classified by the varying pathogeneses observed i.e. tumours (oncoretroviruses), slow, chronic disease (lentiviruses) and the induction of vacuolation in culture (spumaviruses). Current classification systems split the retrovirus family into seven genera, grouped into ‘simple’ viruses (alpha-, beta-, and gammaretroviruses) and ‘complex’ viruses (delta- and epsilonretroviruses, lenti- and spumaviruses), which contain ‘accessory’ and regulatory proteins in addition to the common retroviral structural proteins (section 1.3.2, Fig. 1.1 and Table 1.1).

As well as such exogenous retroviruses, most species possess endogenous retroviruses which integrate into the germline and represent a ‘fossil’ infection. These endogenous retroviruses, such as human endogenous retrovirus K (HERV-K) and pig endogenous retrovirus (PERV), possess the transcription-regulatory long terminal repeat (LTR) sequences and RT activity, and cluster within the betaretrovirus and
gammaretrovirus branches of the *Retroviridae* family tree, respectively (section 1.3 and Fig. 1.1). As this thesis deals specifically with cellular tropism and inhibition of HIV and SIV, only these immunodeficiency viruses will be discussed further.

![Phylogenetic relationship between retroviruses](image)

**Figure 1.1. Phylogenetic relationship between retroviruses.** A neighbour-joining tree based on the RT protein sequences of retroviruses. All seven retrovirus genera are labelled. See Table 1.1 for full virus names and descriptions. Phylogenetic tree courtesy of Dr. David Griffiths, Wohl Virion Centre, Windeyer Institute of Medical Sciences, University College London.

### 1.1.3 Phylogeny of primate lentiviruses

There are three groups of HIV-1 based upon phylogenetic clustering, termed ‘M’ (main), ‘O’ (outlier) and ‘N’ (new)(Fig. 1.2)(Simon *et al.*, 1998a). Group M is responsible for the majority of global infections, and is further divided into 11 recognised subtypes, or ‘clades’, clustering into independent phylogenetic branches based upon the genetic sequence of the *gag* and *env* genes (Fig. 1.2). These are identified by the letters A-D, F-H, J and K, and are approximately 25-35% divergent. Clades A and F possess further subtypes, A1 and A2, and F1 and F2, which are more
similar to each other than other clades (Gao et al., 2001; Triques et al., 2000). The most common subtypes globally are, in descending order, subtypes C, A and then B (Peeters and Sharp, 2000; Thomson, Perez-Alvarez, and Najera, 2002; UNAIDS/WHO, 2002a).

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Examples</th>
<th>Host species</th>
<th>Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpharetrovirus</td>
<td>Rous sarcoma virus (RSV)</td>
<td>Chicken</td>
<td>C-type</td>
</tr>
<tr>
<td></td>
<td>Avian leukosis virus (ALV)</td>
<td>Birds</td>
<td></td>
</tr>
<tr>
<td>Betaretrovirus</td>
<td>Mason-Pfizer monkey virus (MPMV)</td>
<td>Primate</td>
<td>D-type</td>
</tr>
<tr>
<td></td>
<td>Jaagsiekte sheep retrovirus (JSRV)</td>
<td>Sheep</td>
<td>D-type</td>
</tr>
<tr>
<td></td>
<td>Mouse mammary tumour virus (MMTV)</td>
<td>Mouse</td>
<td>B-type</td>
</tr>
<tr>
<td>Gammmaretrovirus</td>
<td>Murine leukaemia virus (MuLV)</td>
<td>Mouse</td>
<td>C-type</td>
</tr>
<tr>
<td></td>
<td>Feline leukaemia virus (FeLV)</td>
<td>Cat</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gibbon ape leukaemia virus (GaLV)</td>
<td>Gibbon</td>
<td></td>
</tr>
<tr>
<td>Deltaretrovirus</td>
<td>Bovine leukaemia virus (BLV)</td>
<td>Cow</td>
<td>C-type</td>
</tr>
<tr>
<td></td>
<td>Human T-lymphotropic virus (HTLV) -1, -2</td>
<td>Human</td>
<td></td>
</tr>
<tr>
<td>Epsilonretrovirus</td>
<td>Walleye dermal sarcoma virus</td>
<td>Fish</td>
<td></td>
</tr>
<tr>
<td>Lentivirus</td>
<td>Human immunodeficiency virus (HIV)-1, -2</td>
<td>Human</td>
<td>Conical core</td>
</tr>
<tr>
<td></td>
<td>Simian immunodeficiency virus (SIV)</td>
<td>Primate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Feline immunodeficiency virus (FIV)</td>
<td>Cat</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Equine infectious anaemia virus (EIAV)</td>
<td>Horse</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Visna/maedi virus (VMV)</td>
<td>Goat/Sheep</td>
<td></td>
</tr>
<tr>
<td>Spumavirus</td>
<td>Primate foamy virus (PFV)</td>
<td>Primates</td>
<td>Immature</td>
</tr>
<tr>
<td></td>
<td>Feline foamy virus (FFV)</td>
<td>Cat</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bovine foamy virus (BFV)</td>
<td>Cow</td>
<td></td>
</tr>
<tr>
<td>Endogenous</td>
<td>Human endogenous retrovirus K (HERV-K)</td>
<td>Human</td>
<td>Defective particles</td>
</tr>
<tr>
<td>retroviruses</td>
<td>Pig endogenous retrovirus (PERV)</td>
<td>Pig</td>
<td>C-type</td>
</tr>
</tbody>
</table>

Table 1.1. The retrovirus family. Summary of the genera within the Retroviridae family, including host species, present classifications, examples and core morphology.

In addition to these 9 subtypes, there exist many variants formed upon coinfection by, and recombination between, independent subtypes. Included in this group of circulating recombinant forms (CRFs) is the previously designated subtype E, predominant in Thailand (Thomson, Perez-Alvarez, and Najera, 2002). Although divergent envelope sequences originally classified this as an independent subtype, subsequent sequence analysis identified it as a recombinant between a parental isolate (subtype E) and subtype A, from which a large part of the genome originates. In addition to AE, 14 more CRFs have been identified and added to the subtype

Like HIV-1, HIV-2 is divided into categories based upon gag and env genetic sequences. There are currently 7 subgroups of HIV-2, termed A-G. Subtype A is the most common subtype, and although only one member of each subtype C, E, F and G has been identified thus far, their genetic divergence is sufficient to warrant classification as a separate subtype (Reeves and Doms, 2002; Schim van der Loeff and Aaby, 1999). The simian counterpart, SIV, is classified according to the species of origin, for example SIVsm (sooty mangabeys) and SIVcpz (chimpanzees). HIV-2 is more genetically similar to SIVsm than to HIV-1 (70-85% identity at the DNA level, in

![Figure 1.2 Phylogenetic relationship between HIV/SIV subtypes. Phylogenetic relationship between HIV-1, -2, and SIV subtypes. Adapted from (Andersson et al., 1999).]
comparison to 55% identity to HIV-1), and as such these sequences cluster very closely phylogenetically (Fig. 1.2). Indeed, some HIV-2 strains are more closely related to SIV sequences than to other HIV-2 sequences (Chakrabarti et al., 1987; Guyader et al., 1987; Hirsch et al., 1989).

1.1.4 Epidemiology and global prevalence

1.1.4.1 Global distribution of HIV

In 2002 there were an estimated 5 million new cases of HIV/AIDS and just over 3 million deaths, bringing the total number of HIV/AIDS cases to 42 million (UNAIDS/WHO, 2002a). HIV is a global concern, with each continent demonstrating alarming increases in prevalence each year. Of all reported cases of HIV, high-income areas of Northern America, Western Europe and Australia, are home to less than 4%. Sub-Saharan Africa, however, bears 70% of the HIV-positive population (Table 1.2 and Fig. 1.3)(UNAIDS/WHO, 2002a).

![Figure 1.3. Global distribution of HIV-1. The global distribution of reported HIV cases at the end of 2002. The predominant regional genetic subtype is highlighted in bold red text, and the total number of reported cases of HIV/AIDS are in bold numbers. Adapted from (Thomson, Perez-Alvarez, and Najera, 2002; UNAIDS/WHO, 2002a).](image-url)
South and South-East Asia house the second-largest number of cases, with 14% of the global distribution. Both awareness and prevention programmes have brought positive effects in countries such as Uganda and South Africa, where HIV prevalence in pregnant women under the age of 20 has decreased since 1998. However, cases in the majority of this continent continue to rise, with over 30% of the population being HIV-positive in areas such as Botswana, Swaziland and Zimbabwe (38.8, 33.4 and 33.7%, respectively). Eastern Europe and Central Asia are undergoing an explosion of HIV incidence, with the number of new infections in 2002 resulting in a 25% increase in the total number of reported cases in this area (UNAIDS/WHO, 2002a).

<table>
<thead>
<tr>
<th>Region</th>
<th>HIV cases</th>
<th>Mode of transmission *</th>
<th>Main subtype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sub-Saharan Africa</td>
<td>29.4 million</td>
<td>Hetero</td>
<td>A, B, C, D, F, G, H, J, K, plus groups O and N</td>
</tr>
<tr>
<td>South /South-East Asia</td>
<td>6 million</td>
<td>Hetero, IDU</td>
<td>B, C, E</td>
</tr>
<tr>
<td>Latin America</td>
<td>1.5 million</td>
<td>MSM, IDU, hetero</td>
<td>B, F</td>
</tr>
<tr>
<td>East Asia/Pacific</td>
<td>1.2 million</td>
<td>IDU, hetero, MSM</td>
<td>BC, B</td>
</tr>
<tr>
<td>Eastern</td>
<td>1.2 million</td>
<td>IDU</td>
<td>A, B, C</td>
</tr>
<tr>
<td>Europe/Central Asia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>North America</td>
<td>980 000</td>
<td>MSM, IDU, hetero</td>
<td>B</td>
</tr>
<tr>
<td>Western Europe</td>
<td>570 000</td>
<td>MSM, IDU</td>
<td>B</td>
</tr>
<tr>
<td>North Africa/Middle East</td>
<td>550 000</td>
<td>Hetero, IDU</td>
<td>AG, A, G</td>
</tr>
<tr>
<td>Caribbean</td>
<td>440 000</td>
<td>Hetero, MSM</td>
<td>B</td>
</tr>
<tr>
<td>Australia/New Zealand</td>
<td>15 000</td>
<td>MSM</td>
<td>B</td>
</tr>
</tbody>
</table>

Table 1.2. Global statistics of HIV infection. Total number of reported HIV/AIDS cases, main mode of transmission, and the main HIV-1 subtypes present in regions around the world. The predominant genetic subtype is highlighted in bold text. * Hetero (heterosexual transmission), IDU (intravenous drug use), MSM (men who have sex with men). Adapted from the regional HIV/AIDS statistics from UNAIDS/WHO (Thomson, Perez-Alvarez, and Najera, 2002; UNAIDS/WHO, 2002a).

Although all HIV-1 group M subtypes are found in Africa, most are found in distinct geographical areas (Table 1.2 and Fig. 1.3)(Vidal et al., 2000). For example, subtype C is dominant in Africa and Asia, subtype B isolates are predominant in the Western world and Australia, subtype AE is dominant in South-East Asia, and subtype
A is very common in Eastern Europe and Western Africa, accounting for 80% of all cases in that area (Thomson, Perez-Alvarez, and Najera, 2002; UNAIDS/WHO, 2002a).

While HIV-1 is globally pandemic, HIV-2 is endemic with stable prevalence rates. The global distribution of HIV-2 is much more restricted, limited to its origin in West Africa, Portugal, and areas of the world with links to Portugal, such as Guinea-Bissau, Angola, Mozambique, India and Brazil (Andersson et al., 1999; Reeves and Doms, 2002; Schim van der Loeff and Aaby, 1999; Soriano et al., 2000). HIV-2 subtype A is the predominant subtype (Norrgren et al., 1997; Reeves and Doms, 2002; Soriano et al., 2000). Subtype B, originating in Ghana and the Ivory Coast, accounts for the majority of cases in this area, whereas the remaining subtypes are all found in Western Africa (Reeves and Doms, 2002; Schim van der Loeff and Aaby, 1999).

1.1.4.2 Transmission of HIV

HIV is a blood-borne virus, transmitted via contact with infected blood, semen and breast milk. The most efficient mode of HIV transmission is male-male, or male-female anal sex, with male-female vaginal transmission being 2-3 times more efficient than female-male transmission (Morison, 2001). The likelihood of infection upon unprotected sex with an infected individual is dependent upon many factors, including the presence of pre-existing sexually transmitted diseases (STDs), and the viral load of the infected individual (Morison, 2001). Although AIDS was first identified in the homosexual population, heterosexual sex is now the most common mode of HIV transmission globally. In addition to sexual transmission, HIV spreads parenterally via contaminated needles, a transmission route that is increasingly common in areas under social and economic stress such as Eastern Europe and Central Asia (Table 1.1). In areas where a high proportion of the HIV-positive population are women, such as Sub-Saharan Africa (58%), North Africa (55%) and the Caribbean (50%), vertical transmission from mother to child is common, occurring either in utero, intra-partum, or post-natally via contaminated breast milk (Morison, 2001; Quinn, 1996; Schim van der Loeff and Aaby, 1999; Toth et al., 2001; UNAIDS/WHO, 2002a).

Epidemiological evidence in Sub-Saharan Africa indicates that transmission of HIV is reduced in circumcised males, as the presence of a foreskin correlates with the acquisition of minor abrasions during intercourse, increased numbers of Langerhans cells and a higher risk of harbouring inflammations and other STDs (Bailey, Plummer,
and Moses, 2001; Morison, 2001; Quigley, Weiss, and Hayes, 2001). However, such procedures and the benefits they may provide, particularly in light of the false sense of protection it implies, render this prevention method controversial (Lagarde et al., 2003).

1.1.5 Origin of HIV

The high level of genetic relatedness between HIV and SIV, inferred that this lentiviral infection arose in humans as the result of a zoonotic transfer from primates (Figs. 1.1 and 1.2). Indeed, such zoonoses from monkeys to humans have occurred before, with monkeypox and primate foamy virus (Korber et al., 2000). The sooty mangabey Cercocebus torquatus atys, infected both in captivity and in the wild, is acknowledged as the natural simian host of both HIV-2 and SIVmac in captive macaques. The Asian macaques Macaca mulatta are not naturally infected in the wild, yet infection of all captive macaques can be traced back to contact or exposure to a sooty mangabey (Chen et al., 1996). Evidence for C. atys as the simian host of HIV-2 includes the overlapping geographic distributions and high level of prevalence of SIVsm within the epicentre of the HIV-2 endemic in West Africa, particularly Sierra Leone, Liberia and the Ivory Coast (approximately 40% of the sooty mangabey population)(Gao et al., 1992; Hirsch et al., 1989). The phylogenetic closeness and genomic sequence similarity between HIV-2 and SIVsm, and a plausible route of transmission – sooty mangabeys are commonly kept as household pets and hunted for food – further support this theory (Chakrabarti et al., 1987; Gao et al., 1994; Hahn et al., 2000; Hirsch et al., 1989; Schim van der Loeff and Aaby, 1999; Sharp et al., 2001). The seven HIV-2 subtypes are equally as divergent from each other, and are thus proposed to have developed from multiple sooty mangabey zoonoses, some of which have been successful (the predominant HIV-2 subtype A) and others that have been less so (for example, subtypes C and D, found in very few individuals)(Chen et al., 1996; Gao et al., 1994; Sharp et al., 1994).

The origin of HIV-1 is less well-defined than HIV-2, and remains controversial (Hillis, 2000; Hooper, 2000; Korber et al., 2000; Royal Society, 2001). The most genetically related sequences to HIV-1 are found in the common chimpanzee, Pan troglodytes (Corbet et al., 2000; Gao et al., 1999; Hahn et al., 2000; Peeters et al., 1992; Peeters et al., 1989; Simon et al., 1998a). Of the seven SIVcpz genomes sequenced from chimpanzees infected in the wild, five were isolated from the subspecies P. t. troglodytes (SIVcpzUS, SIVcpzGab1, SIVcpzGab2, SIVcpzCam3 and SIVcpzCam5),
and two from *P. t. schweinfurthii* (SIVcpzANT and SIVcpzTAN1) (Corbet *et al.*, 2000; Gilden *et al.*, 1986; Peeters *et al.*, 1992; Peeters *et al.*, 1989). As observed with HIV-2, there is a large degree of overlap between the natural habitat of *P. t. troglodytes* (central Western Africa) and the origin of HIV-1 subgroup N (solely found in Cameroon) (Charneau *et al.*, 1994a; De Leys *et al.*, 1990; Simon *et al.*, 1998a). SIVcpz sequences are most closely related to subgroup N, yet cluster within the HIV-1 subgroups M and N (Corbet *et al.*, 2000; Simon *et al.*, 1998a). In fact, subgroup N appears to be a recombinant of an HIV-1 subgroup M strain and an SIVcpz strain, thought to have originated from a co-infection and recombination event within a chimpanzee prior to zoonosis (Corbet *et al.*, 2000; Gao *et al.*, 1999; Hahn *et al.*, 2000).

The tight phylogenetic clustering of the *P. t. troglodytes* sequences with HIV-1 subgroup N sequences provides evidence that chimpanzees harbour the source of HIV-1 (Gao *et al.*, 1999; Hahn *et al.*, 2000; Sharp *et al.*, 2001; Simon *et al.*, 1998a). In addition, the diversity between HIV-1 subgroups M, N and O, suggests that three independent zoonotic events occurred (Gao *et al.*, 1999; Hahn *et al.*, 2000; Sharp *et al.*, 2001).

Although it is generally believed that chimpanzees are the natural host of HIV-1, the precise timing and mechanism of SIVcpz introduction into the human population remains unclear and under some debate. The two main proposed mechanisms for zoonosis include a natural transmission, from bites or exposure to blood of infected chimpanzees, or introduction by the use of chimpanzee kidneys in the production of the oral polio vaccine (OPV) in the late 1950s (Society, 2001). The presence of three distinct HIV-1 subgroups suggests that if chimpanzee kidneys were used for OPV production, they must have been contaminated with three individual SIVs from different sources. This piece of evidence casts doubt on the OPV theory, and favours a natural transmission of SIVcpz into the human population (Hahn *et al.*, 2000; Marx, Alcubes, and Drucker, 2001; Rambaut *et al.*, 2001; Sharp *et al.*, 2001).

The earliest documented case of HIV-1 was in 1959 (Nahmias *et al.*, 1986). Phylogenetic analysis of HIV-1 and SIVcpz sequences strongly suggests that diversification within subgroup M occurred after introduction into the human population (Gao *et al.*, 1999; Hahn *et al.*, 2000; Santiago *et al.*, 2002). The rate of mutation required to allow evolution of nine HIV-1 subgroup M clades following introduction into humans suggests that incidences of HIV-1 should have been observed before this.
first reported case in 1959. In addition, molecular evolutionary experiments using full length sequences of a large number of subgroup M isolates and this earliest documented isolate of HIV-1 estimate the common ancestor of subgroup M clades as the early 1930s, if not before (Korber et al., 2000; Salemi et al., 2001; Yusim et al., 2001).

1.2 Pathogenesis and tropism of HIV

1.2.1 HIV Cellular Tropism

After the discovery of HIV-1, it became clear that virus taken from different stages of disease displayed two distinct phenotypes: virus from early stages of disease replicated slowly in PBMCs and produced low numbers of progeny virions (slow/low), and virus from later-stage individuals replicated rapidly and produced high numbers of virions (rapid/high)(Asjo et al., 1986; Fenyo, Albert, and Asjo, 1989). Rapid/high viruses are more cytopathic and induce the formation of large syncytia in PBMC cultures in vitro. These rapid/high, or syncytium-inducing (SI), strains exhibit a preferential tropism for T-cell lines and are termed T-tropic, whereas the less-cytopathic slow/low, or non-syncytium-inducing (NSI), isolates demonstrate tropism for monocyte-derived cells and are thus M-tropic. As disease progresses, the cellular tropism of HIV shifts from M- to T-tropism, and this shift is associated with a poor prognosis (section 1.6.2.5)(Collman et al., 1989; Connor and Ho, 1994; Fenyo et al., 1988; Schuitemaker et al., 1992; Schwartz et al., 1989). When HIV coreceptors were discovered it was found that the distinct cellular tropism of HIV correlated to the use of the 7-transmembrane G-protein-coupled chemokine receptors (7TM GPCR) CCR5 and CXCR4 on CD4+ cell types (section 1.6.2). The expression of CD4 and a coreceptor is not sufficient to guarantee infection by HIV (Dittmar et al., 1997). Indeed, the relative expression levels of both CD4 and coreceptor can determine the extent of ensuing infection, with relative affinities of gp120 for these receptors affecting subsequent infection (Dejucq, Simmons, and Clapham, 1999; Kozak et al., 1997; Platt et al., 1998; Wu et al., 1997b).

1.2.1.1 CD4-positive T-cells

The major in vivo cellular target for HIV is CD4+ T-helper cells (T_H), and these cells are progressively destroyed throughout the course of disease (Dalgleish et al., 1984; Klatzmann et al., 1984a; Levy et al., 1984). There are two types of T_H cells, termed T_H1 and T_H2, classified according to their chemokine receptor expression, and cytokine secretion profiles, and the cell types they provide help to. The apparent switch
in cytokine profiles of infected individuals, where $T_{H1}$ cytokines decrease and $T_{H2}$ responses increase towards the later disease stages, suggest an early destruction of $T_{H1}$ cells (Romagnani, Maggi, and Del Prete, 1994). However, HIV shows a preferential targeting of the $T_{H2}$ and $T_{H0}$ subsets of $CD4^{+}$ lymphocytes over $T_{H1}$ cells (Maggi et al., 1994; Romagnani et al., 1994). The reason behind this subset preference of HIV remains unclear, due to the complex nature of cytokine production, and the fact that other cell types also secrete these cytokines.

The cytokine profiles in infected individuals may also influence HIV tropism, as both CCR5 and CXCR4 are upregulated by type 1 cytokines (such as IL-2 and IFN-$\gamma$), whereas CXCR4 is upregulated and CCR5 down-regulated by type 2 cytokines (IL-4 and IL-10)(Barcellini et al., 1994; Romagnani, Maggi, and Del Prete, 1994). In addition to the T-helper cell subsets, memory T-cells ($CD45^{RO^+}$) are infected more frequently than naïve ($CD45^{RA^+}$) T-cells, although infection of naïve T-cells by CXCR4-using strains in the later stages of disease has been reported (Ostrowski et al., 1999; Roederer et al., 1997; Schnittman et al., 1990; Spina, Prince, and Richman, 1997). The role of this preference for specific T-cell subsets in HIV pathogenesis remains uncertain.

1.2.1.2 Macrophages, monocytes and microglia

Blood and tissue monocyte-derived macrophages express both $CD4$ and CCR5, are susceptible to infection with HIV, and represent one of the major in vivo cellular targets and latent reservoirs (Gendelman and Meltzer, 1989; Meltzer and Gendelman, 1992). Unlike infected T-cells, which release budded virions extracellularly from the cell surface, infected macrophages have a high number of virions retained within intracellular vesicles, a mechanism which may contribute to virus dissemination (Gendelman et al., 1988). Although all M-tropic isolates exploit CCR5, as do most primary T-tropic strains, macrophages do express CXCR4 capable of supporting infection by some CXCR4-using strains (Collman and Yi, 1999; Simmons et al., 1998; Yi et al., 1998). However, not all CXCR4-using viruses can infect macrophages, and although this can be overcome in some cases by increasing HIV receptor expression levels, some virus strains are inherently restricted from replicating in macrophages (Collman and Yi, 1999; Mondor et al., 1998; Platt et al., 1998; Schmidtmayerova et al., 1998; Simmons et al., 1995; Tokunaga et al., 2001; Wu et al., 1997b). The precise nature of this restriction remains unclear, as macrophages remain sensitive to cell-cell
fusion upon cocultivation with T-cells infected with such restricted isolates (Simmons et al., 1995; St Luce et al., 1993). It remains clear, however, that expression of a coreceptor, and the ability of an isolate to exploit it, does not ensure successful infection of macrophages (Dittmar et al., 1997).

Microglia and perivascular macrophages are the major targets for HIV and SIV in the brain, becoming infected by HIV during the early stages of disease (Davis et al., 1992; Kure et al., 1990; Price et al., 1988; Pumarola-Sune et al., 1987; Stoler et al., 1986; Williams et al., 2001). Although infection of astrocytes can occur in the absence of CD4, microglial infection is CD4-dependent and predominantly mediated via CCR5, despite the presence of other coreceptors such as CXCR4 and CCR3 (Albright et al., 1999; Cheng-Mayer et al., 1989; Ghorpade et al., 1998; Gorry et al., 2001; He et al., 1997; Jordan et al., 1991; Lavi et al., 1997; Sharpless et al., 1992; Shieh et al., 1998; Watkins et al., 1990). As seen with SIV, it is the capacity of HIV isolates to infect macrophages that determines neurotropism, although other cellular and viral factors, including Nef, may play a role (Flaherty et al., 1997; Gorry et al., 2001; Korber et al., 1994; Mankowski et al., 1997; Strizki et al., 1996). An increased affinity for CCR5, as well as a decreased dependency on both CD4 and CCR5 may also be predictive of neurotropism (Gorry et al., 2002a).

### 1.2.1.3 Dendritic cells

There are several phenotypically-distinct antigen-presenting dendritic cells (DCs) whose normal biological role is to present antigen to CD4⁺ and CD8⁺ T-cells. Dendritic cell subtypes include the bone marrow-derived plasmacytoid DCs (pcDCs) and myeloid DCs, as well as Langerhans cells in the skin and genital mucosae. These subtypes display few phenotypic markers, and their chemokine receptor expression profiles differ according to the maturation state (CCR5⁺CXCR4⁻ immature cells; CCR5⁻CXCR4⁺ mature cells). Analysis of dendritic cell infection *in vivo* is therefore complex (Piguet and Blauvelt, 2002; Pope, 1999). Some DC subtypes express low levels of CD4 as well as CCR5 and/or CXCR4, and as such have been reported to support HIV replication both *in vitro* and *in vivo* (Bhoopat et al., 2001; Frankel et al., 1997; Langhoff et al., 1991; MacDougall et al., 2002; O'Doherty et al., 1993; Patterson et al., 1999; Pope, 1999; Simonitsch et al., 2000; Tchou et al., 2001; Walsh et al., 1987; Zaitseva et al., 1997; Zambruno et al., 1991).
Certain DC subsets express C-type lectin receptors such as dendritic cell-specific ICAM-3-grabbing non-integrin (DC-SIGN), which form high-affinity interactions with gp120 (Curtis, Scharnowske, and Watson, 1992; Geijtenbeek, Engering, and Van Kooyk, 2002; Geijtenbeek et al., 2000; Turville et al., 2002). This molecule, and its homologue DC-SIGNR (DC-SIGN-related) are reported to enhance HIV infection by trapping virus on the cell surface, or in intracellular endocytic vesicles, and transporting it to the lymph nodes, although there is little evidence for this in vivo (Baribaud, Pohlmann, and Doms, 2001; Geijtenbeek, Engering, and Van Kooyk, 2002; Piguet and Blauvelt, 2002; Pohlmann, Baribaud, and Doms, 2001). In addition to playing a role in HIV dissemination, DC-SIGN binds to and dramatically enhances infection of target cells by Ebola virus, and has recently been reported to be the major receptor for infection of MDMs with Mycobacterium tuberculosis (Baribaud et al., 2002; Simmons et al., 2003; Tailleux et al., 2003).

Early reports demonstrated a productive infection of germinal centre FDCs in vitro and in situ, but these cells are not believed to be productively infected in vivo (Fujiwara et al., 1999; Parmentier et al., 1990; Schmitz et al., 1994; Spiegel et al., 1992; Sprenger et al., 1995). Their role in the pathogenesis of HIV infection is, however, undisputed. HIV binds FDCs between the multiple cell surface processes, with adhesion molecules CD54 (ICAM-1), CD11a (LFA-1), and complement factor 3 implicated (Fujiwara et al., 1999; Joling et al., 1993; Schmitz et al., 1994). This FDC-bound virus, which remains fully infectious for up to 25 days in vitro and up to 9 months in vivo, is retained within the germinal centres of the lymph nodes, where intimate interactions with activated T-cells allow an efficient transfer of virions (section 1.2.2)(Ayehunie et al., 1995; Chakrabarti et al., 1994; Eitner et al., 2000; Hurtrel et al., 1994; Pantaleo et al., 1994b). The long infectivity half-life of FDC-bound virus thus means that FDCs represent a potent reservoir and possible source of rebounding plasma viraemia upon cessation, or failure of antiretroviral therapy (section 1.8.2) (Burton et al., 2002; Grouard and Clark, 1997; Heath et al., 1995; Smith et al., 2001).

1.2.1.4 Infection of CD4-negative cells

Certain HIV and SIV isolates infect CD4-negative coreceptor-expressing cell lines and primary cell types, including PBMCs, astrocytes, endothelial and epithelial cells, Leydig cells and dendritic cells in vitro (Brichacek et al., 1992; Chehimi et al., 1993; Clapham, McKnight, and Weiss, 1992; Edinger et al., 1997b; Endres et al., 1996;
Lafon et al., 1993; Ray et al., 1998; Reeves et al., 1999; Sabri et al., 1999; Stahmer et al., 1991; Willey et al., 2003). Such CD4-independent infection is inefficient compared to CD4-dependent infection. Although a coreceptor alone can be used by CD4-independent strains, the glycosphingolipid galactosylceramide (galactocerebroside, or GalCer) and ganglioside GM3 act as the primary receptor for HIV on cell lines derived from oligodendrocytes (SK-N-MC) and intestinal epithelial cells (HT-29), respectively (Hammache et al., 1998; Harouse et al., 1989; Yahi et al., 1992). Although infection of these cells is inhibited by anti-GalCer mAbs, CD4-independent infection of other cells, such as astrocytes, is not blocked, indicating the use of other receptors (Chapter 4)(Harouse et al., 1991; Moses et al., 1993).

Brain microglia and macrophages are the major cell types targeted in the brain, but there is an increasing body of evidence demonstrating the infection of CD4-negative astrocytes during the early stages of disease, particularly in paediatric AIDS cases (Boutet et al., 2001; Budka, 1990; Dorf et al., 2000; Tornatore et al., 1994a). In vivo infection of astrocytes is restricted to an overexpression of 'early' HIV genes; however, astrocyte infection in vitro is fully productive (Canki et al., 2001; Messam and Major, 2000; Sabri et al., 1999; Saito et al., 1994; Tornatore et al., 1994b; Willey et al., 2003a). The identity of the coreceptor(s) exploited for astrocyte infection remains controversial, and is complicated by chemokine and coreceptor expression profiles that are often age- and donor-dependent (Boutet et al., 2001; Croitoru-Lamoury et al., 2003; Gabuzda et al., 1998; Lavi et al., 1998; Sanders et al., 1998; Willey et al., 2003a). However, it remains clear that CD4-negative cell types can be infected in vivo, although such infection is infrequent and at a low level. Albeit a relatively rare occurrence, the infection of even a small percentage of CD4-negative cells in vivo increases the host cell range targeted by HIV, and may impact on HIV pathogenesis.

1.2.2 Virus dissemination

Diagnosis of HIV+ status often occurs many months after initial infection (section 1.2.3.1), so the precise mechanisms of virus transmission across mucosal barriers and systemic dissemination into lymph nodes and other lymphoid tissues remain elusive. However, it is clear that lymphoid tissues, including lymph nodes, adenoids and tonsils, harbour the majority of virus, and are the early sites of HIV replication (Pantaleo et al., 1993). The identity of the first cell type infected following exposure to HIV remains unclear, but studies with vaginally-inoculated macaques
indicate that 24-hours post-infection, the majority of infected cells are T-cells, yet up to 40% are dendritic cells (Hu, Gardner, and Miller, 2000; Miller and Hu, 1999).

Irrespective of the initial target cell, once exposed to HIV, virus is either cleared from the bloodstream and transported to spleen, liver and lung lymphoid tissue, or transmitted across mucosal surfaces. Dissemination from mucasae to lymph nodes may occur via attachment of virus particles to immature dendritic cells (DCs), such as Langerhans cells within the genital mucosa, possibly via DC-SIGN, although other attachment factors are involved (section 1.2.1.3)(Ayehunie et al., 1995; Barratt-Boyes, Zimmer, and Harshyne, 2002; Blauvelt, Glushakova, and Margolis, 2000; Braathen, 1988; Piguet and Blauvelt, 2002; Rowland-Jones, 1999; Spira et al., 1996; Wu et al., 2002). DC-captured virus could then be transported to the lymph nodes through the draining lymphatic system, potentially in as little as 7 days (Chakrabarti et al., 1994; Eitner et al., 2000; Hurtrel et al., 1994).

Maturation of DCs during lymph node migration allows an interaction with and subsequent activation of resident T-cells, which are then highly susceptible to cell-cell infection with the transported virus in as little as 30 minutes in vitro (Ayehunie et al., 1995; Zoeteweij and Blauvelt, 1998). The lymph node microenvironment of activated CD4^+ T-cells and high levels of chemokines enhances HIV replication and propagates the activation of latently infected resting T-cells (Khatissian, Chakrabarti, and Hurtrel, 1996). Approximately two weeks post-infection, the major cellular reservoir shifts from infected T-cells to uninfected follicular DCs (FDCs) which sequester virus within germinal centres where they contribute to the infection of circulating CD4^+ T-cells and the systemic dissemination of HIV (Chakrabarti et al., 1994; Eitner et al., 2000; Hurtrel et al., 1994; Schacker et al., 2001; Spiegel et al., 1992; Sprenger et al., 1995).

1.2.3 Pathogenesis of HIV

With the exception of feline immunodeficiency virus (FIV), which causes immunodeficiency in cats similar to that observed in HIV-positive humans, immunodeficiency viruses do not induce AIDS-like disease in their natural host (Norley et al., 1999; Pedersen et al., 1987; Willett, Flynn, and Hosie, 1997). Neither sooty mangabeys nor chimpanzees exhibit AIDS-like symptoms upon infection with SIVsm and SIVcpz, respectively, yet zoonotic transmission to an alternative host (SIVcpz to
humans; SIVsm to the Asian macaque *Maccaca mulatta*) results in a highly pathogenic infection (Desrosiers, 1990; Franchini *et al.*, 1987; Rey-Cuille *et al.*, 1998).

Both host and viral factors contribute to the relative pathogenicity and progression of HIV-1 and HIV-2 disease (Cohen, Kinter, and Fauci, 1997; Hogan and Hammer, 2001a; Hogan and Hammer, 2001b; Schacker *et al.*, 1998). Viral factors include tropism and coreceptor use, the ease at which the viral population can escape immune surveillance, and the relative ‘fitness’ of the virus. In addition, host factors such as the HLA haplotypes present in an individual, and the chemokine/chemokine receptor genotype of an individual, particularly CCR5, CCR2 and SDF-1a, play a role (sections 1.2.4 and 1.5.2)(Hogan and Hammer, 2001b; Ioannidis *et al.*, 2001; Mulherin *et al.*, 2003; Paxton, Kang, and Koup, 1998; Smith *et al.*, 1997).

HIV-2 is thought to be less pathogenic than HIV-1, with infected individuals possessing lower viral setpoints and more efficient immune regulation, yet the clinical trends in HIV-2-infected individuals that progress to AIDS are similar to those observed in HIV-1 patients (Brun-Vezinet *et al.*, 1987; Clapham, McKnight, and Weiss, 1992; Reeves and Doms, 2002; Soriano *et al.*, 2000). Irrespective of the rate of disease progression, all HIV-infected individuals undergo three main stages of disease, classified by CD4+$^+$ T-cell counts – acute, asymptomatic and symptomatic infection (Fig. 1.4)(Anon., 1992; Michael and Burke, 1991).

*1.2.3.1 Acute infection*

Early diagnosis of HIV infection is important, as initiation of antiretroviral therapy and opportunistic infection prophylaxis can dramatically reduce the severity of acute infection symptoms, and thus influence the rate of disease progression (Anon., 1992; Apoola, Ahmad, and Radcliffe, 2002; Orenstein, 2002; Schacker, 1997; Vanhems, Lecomte, and Fabry, 1998). Although 50-70% of HIV-infected individuals exhibit mild clinical symptoms, the primary diagnosis of acute infection, by the presence of virus in the plasma, an absence of HIV-specific antibodies and an extremely high rate of viral replication, remains difficult (Anon., 1992; Apoola, Ahmad, and Radcliffe, 2002; Schacker, 1997; Tindall and Cooper, 1991; Vanhems, Lecomte, and Fabry, 1998). During acute infection CD4+$^+$ T-cell counts are reduced and viral load peaks to as high as $1 \times 10^7$ viral RNA copies per ml of plasma; however, early immune responses result in a control of virus replication, a reduction of viral load and rebound of
CD4$^+$ T-cell counts to high levels (>500 cells/l) (Fig. 1.4) (Anon., 1992; Apoola, Ahmad, and Radcliffe, 2002; Fauci et al., 1996; Perlmutter, Glaser, and Oyugi, 1999; Schacker et al., 1998; Tindall and Cooper, 1991).

**Figure 1.4. Dynamics of HIV replication during disease progression.** The dynamics of viral replication and T-lymphocyte immune responses during the three stages of HIV infection — acute, asymptomatic and symptomatic infection. Adapted from (Fauci et al., 1996)

### 1.2.3.2 Asymptomatic infection

Following the initial burst of viraemia and stimulation of host immune responses, CD4$^+$ T-cell levels and plasma viral load reach equilibrium and HIV infection becomes clinically latent (Fig. 1.4) (Anon., 1992; Michael and Burke, 1991; Royce et al., 1991). Viral setpoint during this stage is highly prognostic, with high plasma viral loads determining the CD4$^+$ T-cell count and subsequent rate of disease progression (Mellors et al., 1997). In general, adults maintain a low viral load and high CD4$^+$ numbers, yet infected children present with high viraemia irrespective of CD4 counts (Saag et al., 1991). CD8$^+$ cell numbers fall after the initial increase, yet remain at a higher level to that seen in primary infection (section 1.2.4) (Anderson et al., 1991). The duration of this asymptomatic period in HIV-1-infected individuals can range from 2 years (rapid progressors) to beyond 10 years [long-term non-progressors (LTNPs); section 1.2.3.4], but on average last 8-10 years (Chevret et al., 1992; Farzadegan et al., 1996; Fauci et al., 1991; Haynes, Pantaleo, and Fauci, 1996; Lifson et al., 1991; Phair et al., 1992; Royce et al., 1991). The asymptomatic period in HIV-2-infected individuals
is longer, lasting over ten years on average, with many individuals never progressing to AIDS (Poulsen et al., 1997; Reeves and Doms, 2002).

Although HIV is clinically latent at this time, patients undergo an accumulative destruction of lymphoid tissue, correlating with progressive immunodeficiency (Pantaleo et al., 1993). In the later stages of the asymptomatic period, when viral load increases and CD4⁺ T-cell counts drop below 500 cells/μl, patients present with clinical symptoms characteristic of a depressed immune system, collectively termed AIDS-related complex (ARC) (Albrecht, 1997; Anon., 1992; Cohen and Fauci, 2001; Michael and Burke, 1991; Orenstein, 2002). Peripheral neurological disorders are also seen during asymptom in approximately 60% of HIV⁺ patients, which increase in severity during the later stages of disease (see below) (Williams and Hickey, 2002).

1.2.3.3 Symptomatic infection and AIDS

The rapid increase in plasma viral load, in combination with the continuing decrease in CD4⁺ T-cell numbers to below 200 cells/μl signals the start of symptomatic infection and the onset of AIDS (Allain et al., 1986; Anon., 1992; Michael and Burke, 1991). Consequently, the severely immunosuppressed patient is highly susceptible to life-threatening new infections, or a reactivation of existing infections, and death is usual within 3-4 years of the onset of symptoms (Michael and Burke, 1991). As well as being susceptible to protozoal, fungal, viral and bacterial infections, AIDS patients display a higher frequency of certain cancers, such as aggressive B-cell lymphomas, non-Hodgkins’ lymphomas, invasive cervical carcinomas and the vascular skin tumour Kaposi’s sarcoma (KS), one of the most common HIV diagnostic diseases alongside Pneumocystis carinii (Boshoff and Weiss, 2002; Carbone, 2003; Phair et al., 1990).

The brain and central nervous system (CNS) harbour HIV from an early stage of infection in approximately 60% of HIV⁺ individuals, and the neurological impairments that ensue are termed HIV-associated dementia (HAD) or AIDS-dementia complex (ADC). ADC is pathologically characterised by widespread astrogliosis (proliferation and hypertrophy of astrocytes), an accumulation and chronic activation of macrophages, production of multinucleated giant cells (MNGCs) and neurodegeneration, and is observed in approximately 30-40% of HIV⁺ individuals during the later symptomatic stages of disease (Budka, 1990; Chiodi and Fenyo, 1991; Gabuzda and Hirsch, 1987; Glass et al., 1995; Levy et al., 1985; Lipton, 1998; McArthur, 1987; Navia et al., 1986;
Navia, Jordan, and Price, 1986; Wiley et al., 1986; Williams and Hickey, 2002). The manner in which the integrity of the protective blood-brain barrier (BBB) is compromised to permit brain and CNS invasion by HIV is not clear, although microglial- and astrocyte-released chemokines may stimulate infiltration of infected monocyte-derived macrophages (section 4.3.2)(Cheng-Mayer et al., 1989; Persidsky et al., 1999; Persidsky et al., 1997).

Neurons themselves are not infected with HIV, but the presence of activated monocytes, microglia and reactive astrocytes contribute to neurodegeneration. The high levels of secreted cytokines from these cells, such as monocyte chemotactic protein 1 (MCP-1), regulated upon activation, normal T-cell expressed and secreted (RANTES), macrophage inflammatory protein 1α and 1β (MIP-1α/MIP-1β), and tumour necrosis factor α (TNF-α), induce the infiltration of blood monocyte-derived macrophages. Both the increase in levels of cytokines and number of infected/uninfected monocyte-derived cells in the brain as disease progresses directly correlate with the onset of ADC/HAD (Brack-Werner, 1999; Chiodi, Britton, and Elovaara, 1996; Conant et al., 1999; Gabuzda et al., 1998; Garden, 2002; Gartner, 2000; Glass et al., 1995; Koenig et al., 1986; Lane et al., 1996; Nuovo and Alfieri, 1996; Persidsky et al., 1999; Schmidt Mayerova et al., 1996; Stevenson and Gendelman, 1994; Williams and Hickey, 2002). Microglia release other factors that interact in positive feedback loops to induce extensive neuronal damage (Lipton, 1998). Some factors [TNF-α, IL-1β, cysteine, and platelet-activating factor (PAF)] activate voltage-dependent N-methyl-D-aspartate (NMDA) receptor-operated channels on neurons, which increases intracellular calcium levels and induces free radical and glutamate release. The normal astrocyte function of mopping up excess glutamate is abrogated by arachadonic acid, also released from activated monocytes/macrophages, and the subsequent high level of glutamate and free radicals induces neuronal death (Lipton, 1998). In addition, HIV-1 proteins including gp120, Tat, and Nef, activate macrophages and are potentially neurotoxic (Dreyer and Lipton, 1995; Ghorpade et al., 2001; Nath, 2002).

1.2.3.4 Long-term non-progressors

Within the HIV+ population exists a small proportion (<5%) of individuals experiencing a prolonged asymptomatic stage of infection (on average >10 years, and up to 15 years), and showing no signs of progression to AIDS (Buchbinder et al., 1994;
Easterbrook, 1994; Fauci et al., 1996). Such individuals demonstrate a consistently high CD4\(^+\) T-cell count and low plasma viral load, even in the absence of antiretroviral therapy, and, unlike patients undergoing disease progression, their lymphoid tissue structure remains intact despite the ability to culture replication-competent virus (Fauci et al., 1996; Pantaleo et al., 1995). Factors contributing to this prolonged asymptomy remain ill-defined. The genotype of the major HIV coreceptor CCR5 is one important factor (section 1.6.2.2)(Carrington et al., 1999; Marmor et al., 2001; Paxton, Kang, and Koup, 1998; Paxton et al., 1996). Virological features also play a role, as individuals infected with a naturally attenuated virus containing deletions within nef demonstrate a slower rate of disease progression (Greenough, Sullivan, and Desrosiers, 1999; Kirchhoff et al., 1995; Learmont et al., 1995). LTNPs have also been shown to have a low viral load upon infection and throughout infection, as well as having much higher neutralising antibody responses against p24, lower levels of CD38\(^+\) CD8\(^+\) T-cells, and CD8\(^+\) T-cell responses directed against Env and, to a lower level, Gag and Nef (Barker et al., 1998; Buchbinder and Vittinghoff, 1999; Harrer et al., 1996; Hogervorst et al., 1995; Pantaleo et al., 1995; Paroli et al., 2001).

1.2.3.5 Exposed seronegative individuals

As well as HIV seropositive rapid progressors and non-progressors, a number of individuals appear to be resistant to infection with HIV-1. Such multiply exposed seronegative individuals (ESNs) include infants of HIV-positive mothers/parents, injecting drug-users, HIV-negative partners in discordant couples, and African sex-workers (Bryson et al., 1995; Fowke et al., 1996; Rowland-Jones et al., 1995; Rowland-Jones et al., 1993). A possible contributor to resistance in the Caucasian population is homozygosity for the 32 base-pair deletion in CCR5 (see above); however, ethnic restrictions, and the fact that not all ESN individuals possess this genotype, demonstrates the role of other factors (Dean et al., 1996; Dorrell et al., 2000; Liu et al., 1996; Makedonas et al., 2002; Marmor et al., 2001; Martinson et al., 1997; Plummer et al., 1999; Rowland-Jones and McMichael, 1995; Samson et al., 1996; Stranford et al., 1999; Yang et al., 2002).

One such factor is an IgA, as detected in the vaginal fluid of ESN African prostitutes (Devito et al., 2000; Dorrell et al., 2000); however, other immune factors must play a role in this resistance. The presence of PBMCs secreting IL-2 in response to HIV-specific T-helper cell epitopes in ESNs indicates a $T_{H1}$ immune response has
occurred that may be protecting some individuals against infection (Fowke et al., 2000; Fowke et al., 1996; Rowland-Jones and McMichael, 1995). In addition, sex-workers in Africa, and other highly sexually-exposed individuals, have much higher MHC-class I restricted cytotoxic and non-cytotoxic CD8^+ T-cell responses (Fowke et al., 2000; Rowland-Jones et al., 1995; Stranford et al., 1999). However, other studies carried out on cohorts of predominantly homosexual ESNs do not find higher HIV-specific CD4^+ and CD8^+ T-cell responses (Schmechel et al., 2001; Yang et al., 2002). The high degree of variability between CTL and T_H cell responses, as well as the chemokine and chemokine receptor profiles in both infected and ESN individuals, means that the exact nature of HIV resistance remains undetermined.

1.2.4 Immune responses to HIV

Although an HIV-specific immune response is mounted following infection, the basic nature of HIV replication i.e. its infection of and progressive destruction of CD4^+ T-cells, results in the decimation of the host immune system to the extent that otherwise non-fatal infections become life-threatening. The reasons behind the initial success of, and eventual failure of the host immune system remains unclear, but it is a complex situation under the control of many host and virus-specific factors (sections 1.2.3.4 and 1.2.3.5).

1.2.4.1 Cell-mediated immunity

Upon initial infection with HIV, CD8^+ T-cells exert both cytotoxic and non-cytotoxic anti-HIV effects. CD8^+CD28^+ T-cells secrete a suppressive factor known as CD8^+ T-cell antiviral factor (CAF), which reduces viral replication in both T-cells and macrophages (Barker et al., 1997; Levy, Mackewicz, and Barker, 1996; Mackewicz and Levy, 1992; Moriuchi et al., 1996; Walker et al., 1986; Wiviott, Walker, and Levy, 1990). Recent publications identify components of CAF as members of the α-defensin family, short antibacterial peptides released from neutrophils, B-cells and NK cells, in addition to CD8^+ T-cells (Zhang, L. et al., 2002). However, the major response against HIV is a broad MHC class I-restricted cytotoxic CD8^+CD28^+ T-cell response (CTL), directed against the polyproteins Gag, Env, and Pol, as well as to their constituent proteins, which successfully reduces viral load and controls viraemia (Borrow et al., 1994; Fiorentino et al., 1996; Koup et al., 1994; Kundu and Merigan, 1992; Ogg et al., 1999; Pantaleo et al., 1997a; Plata, 1989; Riviere et al., 1989). Both the cytotoxic and non-cytotoxic CD8^+ T-cell responses are associated with a slower rate of disease
progression and, in some instances, protection against infection (Borrow et al., 1997; Gomez, Smaill, and Rosenthal, 1994; Greenough et al., 1997; Harrer et al., 1996; Kaul et al., 2001a; Kaul et al., 2001b; Landay, Mackewicz, and Levy, 1993; Pantaleo et al., 1997b; Rowland-Jones et al., 1995). The appearance of Tat and Rev-specific CTLs has also been associated with viral clearance and slow disease progression in humans and macaques (Addo et al., 2001; Geretti et al., 1999; van Baalen et al., 1997).

Although the initial immune response is often vigorous, HIV eventually escapes HIV-specific CTL suppression and individuals progress to AIDS (Borrow and Shaw, 1998; Gea-Banacloche et al., 2000; Rinaldo et al., 1998). The strength and duration of CTL control is dependent upon host and viral factors, including certain MHC class I alleles and T-cell receptor families (such as the β family) associated with more potent anti-HIV activity (Haynes, Pantaleo, and Fauci, 1996; Pantaleo et al., 1994a). In addition, CD4+ T-cell help plays an important role in regulating infection, by exerting MHC class II cytotoxic responses and secreting inhibitory chemokines (Clerici and Shearer, 1994; Garzino-Demo et al., 1998; Gazzinelli et al., 1992; Heeney, 2002; Kalams et al., 1999; Maggi et al., 1987; Pitcher et al., 1999). The loss of T-cell help as disease progresses may also contribute to the decline in anti-Gag antibodies induced early in infection (Binley et al., 1997). It is unclear why host CTL responses eventually fail to control HIV replication, but down-modulation of cell surface MHC-I expression by HIV accessory proteins (Vpu or Nef) may prevent CTL lysis of infected cells. In addition, CTL escape mutants will possess a replicative advantage, and new CTLs specific for these escape epitopes may be less able to control replication (Klenerman, Wu, and Phillips, 2002).

1.2.4.2 Humoral immunity

Antibodies against HIV, in particular anti-gp120, anti-gp41, anti-p24 and anti-p17 antibodies, are detected early upon infection and control initial viraemia. There is thus an association between disease progression and a decrease in plasma levels of these antibodies (Albert et al., 1990; Arendrup et al., 1989; Hogervorst et al., 1995; Keet et al., 1994; Loomis-Price et al., 1998; McRae et al., 1991; Sei et al., 1988; Weber et al., 1987; Zwart et al., 1994). Although most anti-gp120 monoclonal antibodies (mabs) in HIV+ serum recognise epitopes dependent upon the glycosylation pattern within this protein, antibodies recognising the conformational epitope of the CD4-binding site are also induced (Kang et al., 1991; Moore et al., 1990; Posner et al., 1991; Weiss et al.,
1988). Due to the importance of CD4 for HIV infection, the CD4-binding site within gp120 is highly conserved and as such, mabs recognising this epitope neutralise a diverse range of primary and T-cell line adapted isolates (Steimer et al., 1991; Trkola et al., 1995).

In addition to CD4-binding site mabs, antibodies directed against the third hypervariable loop (V3) within the envelope are commonly seen; however, unlike the CD4-binding site mabs, V3-specific mabs are highly strain-specific (Gaines et al., 1987; Goudsmit et al., 1988; Legrand et al., 1997; Page et al., 1992). Conserved sequences located in the crown of the V3 loop (GPXR) represent a major neutralisation epitope, and although changes at this point do affect sensitivity to some V3 mabs, other reports suggest sensitivity is conformation-dependent, and affected by regions outside of this domain (Cecilia et al., 1998; Freed, Myers, and Risser, 1991; Ly and Stamatatos, 2000; McKnight et al., 1996; McKnight et al., 1995; Moore et al., 1995; Poignard, Klasse, and Sattentau, 1996). As such, V3 mab neutralisation is independent of HIV coreceptor-use (Cecilia et al., 1998; LaCasse et al., 1998).

Broadly-reactive antibodies against the transmembrane envelope subunit gp41, as well as against conformational sites exposed upon CD4-binding (CD4-inducible, or CD4i epitopes) are present after infection with HIV (Moore and Sodroski, 1996; Muster et al., 1993; Thali et al., 1993; Wu et al., 1996). Due to the conformational nature of their epitopes, such CD4i mabs are broadly-reactive, and, like CD4-binding site-directed mabs, they inhibit gp120:CCR5 interactions (Raja et al., 2003; Sullivan et al., 1998; Trkola et al., 1996a; Trkola et al., 1996b; Xiang et al., 2002). Although the well characterised gp41-specific neutralising mab 2F5 is directed against a well-conserved linear epitope within the ectodomain of gp41, is still possesses a broad neutralising capacity (Parker et al., 2001).

Neutralising mabs isolated from infected individuals are effective at neutralising HIV in vitro, but are less effective against primary patient isolates (McKnight et al., 1992; Montefiori et al., 1996; Moog et al., 1997; Sullivan et al., 1995; Zwart et al., 1994). This is in part due to protection of the CD4 binding site by envelope glycosylation (Ly and Stamatatos, 2000). In addition, the conformational epitopes recognised by neutralising mabs are likely to be different in primary as compared to lab-adapted isolates, leading to a differential exposure of binding sites and/or V3 loops and
thus varying sensitivity to neutralisation (Bou-Habib et al., 1994; Kwong et al., 2002; Sullivan et al., 1995; Wei et al., 2003). The observation that CD4-independent HIV and SIV isolates, whose envelopes exist in a 'looser' conformation with coreceptor binding sites already partially or fully exposed, are more sensitive to neutralisation supports this notion (Kolchinsky, Kiprilov, and Sodroski, 2001; Puffer et al., 2002; Thomas et al., 2003). As broadly reactive neutralising mabs are slow to develop within most individuals, virus is only weakly neutralised in vivo, and thus nabs are thought to play only a minor role in the early control of viral replication (Pellegrin et al., 1996; Pilgrim et al., 1997; Poignard et al., 1999).

As well as neutralising infection, HIV-specific antibodies mediate antibody-dependent cell-mediated cytotoxicity (ADCC), whereby gp120- or gp41-specific mabs bind to IgG Fc receptors on infected cells and enable killing by natural killer cells and monocytes (Evans et al., 1989; Lyerly et al., 1987; Ojo-Amaize et al., 1987; Tyler et al., 1989). These ADCC-mediating antibodies are present throughout disease, and like neutralising mabs, decreasing titres are associated with progression to disease (Ljunggren et al., 1989).

1.3 Immunodeficiency Virus Genomes

1.3.1 Genomic organisation

As a typical retrovirus, HIV has an RNA genome containing the common structural genes gag, pol and env. Unlike previously sequenced 'simple' retroviruses (such as Rous sarcoma virus), the 'complex' immunodeficiency viruses HIV and SIV possess an additional 6 open reading frames (ORFs) within the 9.7 kb genome encoding two regulatory proteins (Tat and Rev) and four 'accessory' proteins (Nef, Vif, Vpr and Vpu). The HIV-1 and SIVcpz genomes contain the accessory gene vpu, whereas HIV-2 and SIV contain vpx (section 1.3.2 and Table 1.3). The HIV genome is flanked by identical sequences known as long terminal repeats (LTRs), which possess two unique untranslated regions (U3, approximately 450 bp, and U5, approximately 80 bp) and a central repeated region (R) of approximately 100 bp (Fig. 1.5). These LTRs contain elements required to control transcription of the HIV genome in conjunction with the regulatory proteins Tat and Rev (section 1.3.2.2 and 1.5.2). The initial transcription events produce multiply-spliced transcripts (1.7-2 kb) encoding the Tat, Rev and Nef proteins, with later transcription producing partially spliced (4.3-5.5 kb) and unspliced transcripts encoding Vif, Vpu, Vpr and Env, Gag and Gag-Pol. Following translation,
the Env, Gag and Gag-Pol polyproteins are processed by cellular (Env) and viral (Gag and Gag-Pol) proteases, to produce the constituent viral proteins (Fig. 1.5)(Frankel and Young, 1998; Freed and Martin, 2001).

Figure 1.5 Genomic organisation of HIV and processing of viral proteins. Genomic organisation of HIV-1/SIVcpz (A) and HIV-2/SIV (B). Light grey shading (■) represents genes encoding structural proteins; dark grey shading (▲) indicates regulatory genes; very pale grey (□) represents accessory genes. The LTR domains are represented as shaded boxes (U3 □; U5 □; R □).
1.3.2 Gene products of HIV and SIV

1.3.2.1 Regulatory proteins (Tat and Rev)

**Tat**

The *tat* gene (transactivator of viral transcription) contains two exons, and *in vivo* either a one-exon [72 amino acids (aa)] or a two-exon (101 aa) protein is found. As both functional domains of Tat are located in the first exon, this alone is sufficient for the transcription-enhancing activity of HIV-1; however, the second exon in HIV-2 Tat increases binding affinity for the transactivating response (TAR) structure (Rhim and Rice, 1994). The basic, arginine-rich region binds the stem-loop TAR structure, and the N-terminal activation domain contains proline-rich and cysteine-rich domains which recruit cellular factors to stabilise the TAR (Rappaport et al., 1989; Weeks et al., 1990). Tat enhances transcription from the 5' LTR approximately 100-fold by binding to the crucial RNA hairpin structure, the TAR, and aiding the formation and stabilisation of an efficient RNA pol II translation complex (section 1.5.2.3)(Laspia, Rice, and Mathews, 1989). Tat also has potent angiogenic effects, and is thought to contribute to the highly vascularised lesions characteristic of KS by interacting with cell surface receptors (Watson and Edwards, 1999).

**Rev**

Rev (regulator of viral protein expression) is a 116 aa 2-exon protein which regulates the transport of unspliced viral mRNAs by binding to the specific sequence and secondary structure located within the *env* coding region termed the Rev response element (RRE) (Malim et al., 1989; Zapp and Green, 1989). The arginine-rich region of Rev binds to the purine-rich stem-loop II within the complex, multiple-stem-loop structure of the RRE, where it recruits additional Rev molecules. The leucine-rich nuclear export sequences (NES) in the hydrophobic domain of Rev subsequently interact with nuclear export machinery to allow the export of RRE-containing partially spliced or unspliced mRNAs for translation in the cytoplasm (section 1.5.2.4).

1.3.2.2 Structural proteins (Gag, Pol and Env)

**Gag**

The group-specific antigen (Gag) polyprotein is translated from the unspliced mRNA transcript as Pr55Gag or as the fusion protein Pr160Gag-Pol, produced by a ribosomal frameshift occurring during translation of Pr55Gag. Once translated, Gag multimerises and is targeted to the cell membrane. Following virion assembly and
particle release, Gag is processed into its four constituent proteins [matrix (MA), capsid (CA), nucleocapsid (NC) and p6] and two spacer peptides (p1 and p2) by the viral PR, cleaved from the Gag-Pol polyprotein (section 1.5, and Figs. 1.5 and 1.6).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Size (kb)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tat</td>
<td>p14</td>
<td>Enhances transcription.</td>
</tr>
<tr>
<td>Rev</td>
<td>p19</td>
<td>Regulates RNA splicing.</td>
</tr>
<tr>
<td>Matrix, MA</td>
<td>p17</td>
<td>Cleaved from the N-terminus of the Gag polyprotein. Lines the inside of the virion and targets Gag/Gag-Pol to the membrane before assembly.</td>
</tr>
<tr>
<td>Capsid, CA</td>
<td>p24</td>
<td>Forms the viral core and has roles in assembly and uncoating events.</td>
</tr>
<tr>
<td>Nucleocapsid, NC</td>
<td>p7</td>
<td>Coats the viral RNA and delivers it to the virion during assembly.</td>
</tr>
<tr>
<td>p6</td>
<td>p6</td>
<td>Essential for virion incorporation of Vpr and efficient virion budding.</td>
</tr>
<tr>
<td>Vpu</td>
<td>p16</td>
<td>An integral membrane protein that enhances virus release and degrades CD4 within the ER.</td>
</tr>
<tr>
<td>Nef</td>
<td>p27</td>
<td>Lowers cell surface CD4 and MHC-I levels by inducing down-modulation and lysosomal degradation.</td>
</tr>
<tr>
<td>Protease, PR</td>
<td>p10</td>
<td>Cleaves viral polyproteins Gag and Gag-Pol after virion budding.</td>
</tr>
<tr>
<td>Vif</td>
<td>p23</td>
<td>Crucial for the production of highly infectious virions in a cell type-specific manner.</td>
</tr>
<tr>
<td>Surface, SU</td>
<td>gp120</td>
<td>Mediates virion attachment to target cells via binding to CD4.</td>
</tr>
<tr>
<td>Transmembrane, TM</td>
<td>gp41</td>
<td>Anchors gp120 to the membrane and mediates virus and target cell membrane fusion following gp120:CD4:coreceptor-binding.</td>
</tr>
<tr>
<td>Vpr</td>
<td>p15</td>
<td>Stimulates viral gene expression, transports the PIC complex to the nucleus and induces cell cycle arrest at G2.</td>
</tr>
<tr>
<td>Vpx</td>
<td>p16</td>
<td>HIV-2/SIV-specific and highly homologous to Vpr. Responsible for the infection of non-dividing cells.</td>
</tr>
<tr>
<td>Reverse transcriptase, RT</td>
<td>p66</td>
<td>Catalyses the synthesis of a duplex DNA genome from the viral RNA.</td>
</tr>
<tr>
<td>Integrase, IN</td>
<td>p32</td>
<td>Catalyses incorporation of viral DNA into the host genome.</td>
</tr>
</tbody>
</table>

Table 1.3. Gene products of HIV and SIV. A brief summary of the fifteen HIV/SIV proteins, including size and functions.

MA (p17)

The matrix protein is located at the N-terminus of Gag, and the N-terminal myristate group and basic domain target the Gag and Gag-Pol polyproteins to the cellular
membrane. MA inserts into the cell membrane as a trimer and has roles in the incorporation of the Env polyprotein into the membrane via interactions with the long cytoplasmic tail of gp41. It has been proposed that the classical nuclear localisation signal (NLS) within MA aids in the infection of non-dividing cells by assisting translocation of the viral preintegration complex (PIC) to the nucleus; however, the role of MA in PIC nuclear localisation now appears to be negligible, although a role in the uncoating steps following membrane fusion is still possible (Dorfman et al., 1994; Freed and Martin, 1995; Freed and Martin, 2001; Haffar et al., 2000; Yu et al., 1992).

CA (p24)

Capsid (CA) forms the central core of the HIV virus particle. The N-terminal two-thirds of capsid form the ‘core’ domain, which is highly helical and possesses a proline-rich cyclophilin A-binding loop (Gamble et al., 1996). The C-terminal third of CA contains the ‘dimerization domain’, and allows the CA-CA interactions required for the formation of the viral core. Also within the C-terminal domain of CA is the major homology region (MHR), the most highly conserved region between retroviral Gag proteins, which is required for particle assembly. The role of cyclophilin A (CypA) in the life cycle of HIV remains uncertain. However, the presence of CypA either within the target cell or within the virion (via interactions with CA) appears to enhance infectivity during early post-entry (pre-reverse transcription) events (Braathen, 1988; Franke, Yuan, and Luban, 1994; Saphire, Bobardt, and Gallay, 2002). It is possible that CypA enhances infectivity of HIV, and other lentiviruses, by overcoming virus and species-specific intracellular restrictions to replication, such as those observed with retroviruses and the restriction factors Ref1 and Lv1 (Hatzioannou et al., 2003; Kootstra et al., 2003). Both HIV-2 and SIV CA can bind CypA, yet they do not appear to incorporate it into their virions (Billich et al., 1995; Thali et al., 1994). The relevance of this remains unclear.

NC (p7)

The major role of the nucleocapsid protein is to encapsidate viral genomic RNA and deliver it to the assembling virion. Zinc-finger motifs and a basic domain within NC bind to the four stem-loop structure, located between the 5' LTR and the gag initiation codon known as the packaging signal, or ψ. NC also aids the tight assembly of Gag proteins within the virion (Dawson and Yu, 1998; Zhang, Y. et al., 1998a). The nucleic acid chaperone function of NC has roles in the formation of the genomic RNA dimer
and the binding of the tRNA\textsuperscript{2} primer to the primer-binding site, as well as in the initiation of reverse transcription and strand transfers during reverse transcription (section 1.5.2.1)(Cen \textit{et al.}, 1999; Feng \textit{et al.}, 1999).

\textbf{p6}

The C-terminus of Gag encodes a proline-rich 6-kDa late domain protein termed p6, which has roles during the late stages of virus release, when cellular and viral membranes part. Viruses lacking p6 are unable to completely bud from the host cell, and leave viral particles tethered to the membrane. The protein-binding PTAP motif within p6 is essential for its function, which is likely to involve the recruitment of proteins from intracellular endocytic pathways (Demirov \textit{et al.}, 2002; Freed, 2002; Garrus \textit{et al.}, 2001; Myers and Allen, 2002; VerPlank \textit{et al.}, 2001). p6 also directs incorporation of the accessory proteins Vpr and Vpx of HIV/SIV into the virus particle (Accola \textit{et al.}, 1999; Bachand \textit{et al.}, 1999; Mahalingam \textit{et al.}, 2001; Pancio and Ratner, 1998; Selig \textit{et al.}, 1999).

\textbf{Pol}

The Pol polyprotein is translated as a Gag-Pol product upon an occasional ribosomal frameshift during Gag translation, and becomes part of a p160 Gag-Pol precursor. The constituent proteins of Pol (RT, PR, IN) are synthesised at much lower levels than the structural proteins encoded by Gag. The N-terminal myristate on Gag-Pol targets Pol to the plasma membrane and into virions, where it is cleaved by PR upon virion release.

\textbf{PR (p10)}

The viral protease is responsible for virion maturation events occurring following virion budding, whereby the Gag and Gag-Pol polyproteins are cleaved by a PR dimer to produce the proteins MA, CA, NC and p6 (Gag), and PR, IN and RT (Pol)(section 1.5.4). Premature cleavage of Gag and Gag-Pol precursors results in the production of defective virions, so the timing of PR activation is crucial. Initial PR activity is dependent upon the relative amounts of translated Gag versus Gag-Pol proteins, as well as the rate of auto-processing to release PR from Gag-Pol, which may be influenced by p6 (Yu \textit{et al.}, 1998). It is currently unclear whether autocatalysis of Gag-Pol by PR occurs intermolecularly or intramolecularly. PR also cleaves the accessory protein Nef, but the role of this in Nef function remains undetermined. The absolute requirement of
PR activity for the formation of infectious virus particles made it a prime target for antiretroviral drugs; indeed, PR inhibitors have revolutionised antiretroviral therapy (section 1.8.2).

**RT (p66)**
Reverse transcriptase is an enzyme that carries out the three enzymatic functions required to synthesise double-stranded DNA from a single-stranded RNA template: reverse transcription (synthesis of DNA from RNA), DNA polymerase activity (synthesis of dsDNA from a ssDNA template) and ribonuclease H activity (degradation of RNA from the DNA:RNA hybrid)(section 1.5.2.1 and Fig. 1.8). RT is active as a heterodimer, consisting of a p51 and a p66 subunit.

**IN (p32)**
Integrase is cleaved from the C-terminal end of Gag-Pol, and catalyses a series of reactions culminating in the integration of the viral DNA into the host cell genome (section 1.5.2.2). This integrated provirus acts as a template for the production of viral RNAs for the life of the infected cell, and subsequent generations. IN has three functionally distinct domains. The catalytic central core domain is well conserved among retroviral integrases and although it cannot catalyse the processing and joining reactions of integration, it catalyses a reverse disintegration reaction in the presence of divalent cations. The N-terminal zinc-finger domain aids tetramerisation of IN and stabilisation of the PIC, whereas the non-specific DNA-binding capacity of the C-terminal domain maintains contact of the PIC with the viral genome.

**Env**
The envelope glycoprotein is translated within the rough endoplasmic reticulum (ER) as a gp160 precursor. This integral membrane protein becomes post-translationally glycosylated and trimerised within the ER before it is trafficked to the Golgi apparatus and cleaved by cellular proteases to produce the two non-covalently linked Env subunits SU (gp120/gp105) and TM (gp41/gp35)(HIV-1/HIV-2).

**SU (gp120/gp105)**
Gp120, the surface subunit of Env, is structurally complex and highly glycosylated (section 1.7.1)(Kozarsky et al., 1989). This glycan shield aids the creation of an active envelope conformation, and offers a large amount of protection against host anti-gp120
antibody responses (Fenouillet, Gluckman, and Jones, 1994; Losman et al., 2001). As gp120 is expressed on the surface of the virus particle, it mediates the first contact with the target cell receptors (section 1.5.1 and 1.6.2).

TM (gp41/gp35)
The transmembrane subunit of Env, gp41/gp35, mediates fusion between the viral and host cell membranes. The ectodomain of gp41/gp35 is comprised of a hydrophobic N-terminal domain, or ‘fusion peptide’, which possesses a leucine zipper-like heptad repeat motif and forms a trimeric coiled-coil structure, and a C-terminal domain with an amphipathic helix (section 1.7.4). The cytoplasmic domain of gp41 also possesses an internalization sequence inducing endocytosis, which is suppressed by Pr55<sup>agg</sup> to allow Env incorporation into the budding virions (Egan et al., 1996). Like gp120, the ectodomain of gp41 is glycosylated, albeit to a lower level, and the location of these glycans on the external surface of the fusion-active six-helix bundle of gp41 may help stabilise this structure during fusion (Perrin, Fenouillet, and Jones, 1998).

1.3.2.3 Accessory proteins (Nef, Vif, Vpu/Vpx and Vpr)
Original observations indicated that the four unique lentiviral proteins encoded by the additional ORFs in the HIV genome were not absolutely required for virus replication in vitro, earning them the title of ‘accessory proteins’. However, subsequent studies find they have important roles in the viral life cycle, infectivity of released virions and for HIV pathogenesis.

Nef (p27)
Nef is a myristylated phosphoprotein with pleiotropic roles in the replication and life cycle of HIV (Table 1.3)(Luo, Foster, and Garcia, 1997; Piguet and Trono, 1999). Although originally termed ‘negative factor’ due to observations that it reduced virus replication, it was later demonstrated to enhance virus replication (Aiken and Trono, 1995; Chowers et al., 1994; Miller, Feinberg, and Greene, 1994). Other roles of Nef include CD4 and MHC class I down-modulation and degradation, and a modulation of cellular activation pathways (Garcia and Miller, 1992; Greenway, Azad, and McPhee, 1995; Schwartz et al., 1989). Nef induces CD4 down-modulation by binding to, and thus bridging, CD4 and adapter protein complexes in clathrin-coated pits, as well as binding to and dissociating the cellular kinase p56<sup>ck</sup>, which inhibits CD4 internalisation (Salghetti, Mariani, and Skowronski, 1995).
Vif (p23)

Virus infectivity factor acts at the stage of virus assembly, and is crucial for the production of infectious virus particles in so-called non-permissive cells (such as primary lymphocytes and macrophages) (Gabuzda et al., 1994; Hevey and Donehower, 1994; Kishi et al., 1992). The anti-viral factor suppressed by Vif, present in non-permissive cells, is CEM15, or APOBEC3G, a cytosine deaminating (C→U) RNA-editing protein of the apolipoprotein B mRNA editing catalytic peptide family (Jarmuz et al., 2002; Madani and Kabat, 1998; Sheehy et al., 2002; Simon et al., 1998b; Wedekind et al., 2003). As Δvif particles from non-permissive cells demonstrate a post-entry block to infection and unstable PICs, CEM15 may exert its anti-viral effects by processing viral RNA within the virion, rendering it a poor template for reverse transcription, or by processing cellular genomic RNA and altering a protein required for reverse transcription upon cell entry (Dettenhofer et al., 2000; Goncalves et al., 1996) (Borman et al., 1995; Simon and Malim, 1996). Recent studies suggest that the former is the case, and the presence of Vif in non-permissive cells prevents C→U mutations in the minus-strand of viral DNA during reverse transcription (Lecossier et al., 2003).

Vpr/Vpx (p15)

Viral protein R is incorporated into the assembling virion upon interactions with the leucine-rich domain in the C-terminus of p6, and has roles during the early post-entry stages of the HIV replication cycle (Bukrinsky and Adzhubei, 1999; Fouchier and Malim, 1999; Paxton, Connor, and Landau, 1993). Although Vpr lacks a classical NLS, interactions with nucleic acids and nuclear pore complexes aid its induction of nuclear localisation of the PIC, a function which allows infection of non-dividing cells (monocytes and macrophages) (Karni et al., 1998; Popov et al., 1998; Vodicka et al., 1998; Zhang, S. et al., 1998). As well as facilitating nuclear import, Vpr initiates low-level transcription from the HIV LTR, and induces cell cycle arrest in G2 (Jowett et al., 1995; Subbramanian et al., 1998; Wang et al., 1995). HIV-2 and SIV express viral protein X (Vpx), which shares the role of HIV-2 Vpr, i.e. aids infection of non-dividing cells (Hattori et al., 1990). Like HIV-1, HIV-2 Vpr induces cell cycle arrest (Planelles et al., 1996).

Vpu (p16)

Viral protein U is an integral membrane phosphoprotein unique to HIV-1 and SIVcpz viruses, and its major roles are the degradation of CD4 within the ER, and the increase
of virus release (Klimkait et al., 1990; Willey et al., 1992). When gp160 is translated, it is retained in the ER due to the formation of complexes with newly synthesised CD4. Vpu, also translated in the ER, binds to the cytoplasmic tail of CD4, and targets it for ubiquitin-mediated degradation upon interaction with other cellular proteins (Kimura, Nishikawa, and Ohyama, 1994; Lenburg and Landau, 1993; Schubert et al., 1998). The mechanism by which Vpu enhances particle release is less clear, but it involves the N-terminus, and is non-specific, based upon observations that it also enhances the release of viruses that lack Vpu, such as MuLV and HIV-2 (Deora, Spearman, and Ratner, 2000; Gottlinger et al., 1993; Schubert et al., 1996a). HIV-2/SIV lack Vpu, but its function in enhancing virion release is believed to fall to the HIV-2 env (Bour et al., 1996). In addition to enhancing particle release and degrading CD4, Vpu proteins are proposed to form functional cation channels, although the role of this in Vpu function is not known (Ewart et al., 1996; Schubert et al., 1996b).

1.4 Structure of the HIV Virion

HIV is assembled at the plasma membrane and released as a 110 nm diameter immature, spherical particle, containing unprocessed Gag and Gag-Pol polyproteins. Once released, PR cleaves these structural polyproteins and the virion undergoes internal morphological changes to produce the mature virion. The viral membrane, taken from the host cell, is embedded with trimeric viral envelope spikes, and is internally lined with a sphere of MA and Vpr (Fig. 1.6). The central CA core houses the two copies of the NC-condensed RNA genome, as well as PR, IN and RT.

1.5 Life Cycle of HIV

1.5.1 Attachment, fusion, entry and uncoating

The specific cell-virus attachment events take place between the trimeric envelope spike gp120, and the cellular receptor CD4 (Fig. 1.7, step 1; sections 1.6.1 and 1.7.2)(Dalgleish et al., 1984; Klatzmann et al., 1984b). However, initial adsorption of HIV and other viruses, such as Dengue virus, herpes simplex virus and flaviviruses, onto the target cell occurs via an interaction between the envelope proteins and cell-surface glycosaminoglycans (GAGs), such as heparan sulphate, or mannose-binding proteins (Chen, Y. et al., 1997; De Clercq, 2002; Larkin et al., 1989; Shukla et al., 1999; Su et al., 2001). With HIV, the positively-charged V3 loop of gp120 binds the GAGs (Gallaher et al., 1995; Moulard et al., 2000). As such, polyanionic compounds, such as polysulfonates, can inhibit adsorption and subsequent infection by HIV and
other viruses (Andrei et al., 1991; Baba et al., 1988; De Clercq, 2002; Luscher-Mattli and Gluck, 1990; Witvrouw and De Clercq, 1997).

![Diagram of HIV virion](image)

**Figure 1.6. Structure of the HIV virion.** Diagrammatical representation of an HIV virus particle, with all HIV proteins labelled. The nucleocapsid core is centralised within the virion, and only represented higher in this figure for diagrammatical purposes.

In addition to CD4, HIV entry requires interactions with a second receptor, or coreceptor (section 1.6.2). This point in the HIV life cycle is of major interest with respect to the development of new HIV therapies (section 1.8.3.1). Contact between gp120 and the coreceptor induces membrane fusion events, mediated by the gp41 fusion peptide in an as-yet ill-defined process that delivers the virus core into the cell cytoplasm (Fig. 1.7, step 2; section 1.7.4). The precise nature of these uncoating events are unclear, but the end result is the loss of CA and conversion of MA, NC, RT, IN,
Vpr, viral RNA and cellular proteins such as the non-histonal chromosomal protein HMG1(Y), into the high molecular weight reverse transcription complex (RTC) (Freed and Martin, 2001) (Fig. 1.7, step 3).

Figure 1.7. Life cycle of HIV. The major stages in the life cycle of HIV from receptor binding to virus particle budding and maturation, indicating points of action of current and future anti-HIV therapies. RTI, reverse transcription inhibitor; PI, protease inhibitor; CI, coreceptor inhibitor; FI, fusion inhibitor; RTC, reverse transcription complex; PIC, pre-integration complex.

1.5.2 Replication of the HIV genome
1.5.2.1 Reverse transcription

Reverse transcription occurs within the RTC following fusion and uncoating (Fig. 1.7, step 4), and is summarised in Fig. 1.8. Conversion of the positive-sense ssRNA genome into dsDNA is initiated by a virion-incorporated tRNA\textsuperscript{lys3} primer, which binds to the primer-binding site (PBS) downstream of the 5 LTR. The RT heterodimer thus synthesises negative-strand DNA to the 5 end of the genome (Fig.
1.8, step 1). The RNA half of this RNA:DNA dimer is degraded by the RNase H activity of RT to produce the minus-strand strong-stop DNA (step 2).

**Figure 1.8. Reverse transcription.** A summary of the seven major steps of reverse transcription. The primer is represented as a dashed black line, the initial primer-binding site (PBS) is in red, RNA is in black, the positive DNA strand is blue, and the negative DNA strand is green: cPPT, central polypurine tract; 3 PPT, 3′ polypurine tract.

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The newly synthesised ‘R’ region of the 5’LTR is transferred to the 3’ end of the genome during the first strand transfer, where it primes the 3’→ 5’ reading, and 5’→ 3’ synthesis, of the minus strand DNA up to the PBS (step 3). The RNA portion of this duplex is again degraded, with the exception of the central and 3’ polypurine tracts (cPPT and 3’PPT), which are retained to prime the synthesis of positive-strand DNA (step 4). The tRNA primer and the two PPTs are degraded, allowing hybridisation of the positive- and negative-strand in the second strand transfer (steps 5 and 6). The synthesis of the positive strand is then completed via this intermediate (step 7). Replication ends at the central termination signal (CTS), which displaces approximately 100 bp to form a triple-stranded structure known as the central DNA flap (Charneau et al., 1994b).

1.5.2.2 Nuclear import and integration

The preintegration complex (PIC) enters the nucleus via the nuclear pore complex without disrupting the nuclear membrane, a unique feature that allows lentiviruses to infect non-dividing cells (Fig. 1.7, step 5). All components of the PIC, as well as cellular proteins such as HMGa1, have had proposed roles in nuclear import, with MA, Vpr and IN possessing NLSs (Bukrinsky et al., 1993; Depienne et al., 2000; Gao et al., 2003; Heinzinger et al., 1994; Sherman and Greene, 2002). However, both Δvpr and ΔMA viruses, as well as N-terminal NLS-deleted MA viruses, are able to infect non-dividing cells (Fouchier et al., 1997; Greber and Fassati, 2003). Although the central DNA flap produced during reverse transcription has been implicated in nuclear import, cPPT mutants are still able to initiate a productive infection, and this region is thus currently thought to play no major role in nuclear localisation/import of the PIC (Dvorin et al., 2002; Limon et al., 2002; Sirven et al., 2000; Zennou et al., 2000). The precise mechanism for the energy-dependent transport of the PIC into the nucleus via the nuclear pores still remains unclear.

Once the PIC enters the nucleus, the HIV DNA genome is integrated into the host cell genome to form the HIV provirus. The integration target sequences within the cell genome are not yet determined; however, integration sites seem to appear within active genes at a higher frequency than in inactive genes (Schroder et al., 2002). The first step of the 3-step integration process is 3’-end processing, where IN cleaves several bases from the 3’ termini of both viral DNA strands. The cellular DNA is then cleaved at the 5’ end, and the viral pre-integration substrate is ligated to these overhanging
termini in the strand-transfer step. Cellular repair enzymes fill in the gaps, leaving a 5-bp direct repeat either side of the HIV provirus, and integration is complete.

1.5.2.3 Transcription

Transcription of the HIV provirus, the template for viral RNA synthesis, initiates at the U3/R junction in the 5' LTR (Fig. 1.7, step 6). The U3 region is rich in cis-acting regulatory elements, such as a TATA element, NF-κB and Sp1 binding sites, which direct the RNA polymerase II to the site of transcription initiation. In the absence of Tat, transcription of the HIV provirus from the LTR is inefficient and only short transcripts (approximately 60 bp) are synthesised (Feinberg, Baltimore, and Frankel, 1991; Kao et al., 1987; Marciniak and Sharp, 1991). Binding of Tat to the TAR element enhances the rate of transcription by recruiting the positive-transcriptional elongation factor b (p-TEF-b) complex. Cyclin T1 of this complex binds to the activation domain of TAR-bound Tat, and enhances both the affinity of Tat for the TAR and the recruitment of the pTEF-b complex (Wei et al., 1998; Zhang et al., 2000). The kinase partner of cyclin T1 within the Tat-bound pTEF-b complex, cyclin-dependent kinase 9 (CDK9), induces hyperphosphorylation of the C-terminal domain of RNA pol II, increases its processivity and thus enhances transcription (Cujec et al., 1997; Fujinaga et al., 1998; Ivanov et al., 1999; Jones, 1997; Okamoto et al., 1996; Parada and Roeder, 1996; Zhou et al., 1998).

1.5.2.4 RNA transport and translation

Once synthesised, the 5'-capped and 3'polyadenylated unspliced, partially-spliced and multiply-spliced viral mRNA species are transported into the cell cytoplasm (Fig. 1.7, step 7). Intron-containing mRNA species are normally retained within the nucleus; however, the binding of Rev molecules to the RRE allows an interaction with the importin-β family nuclear export machinery via the NES within Rev (Fischer et al., 1995; Fritz and Green, 1996; Meyer and Malim, 1994; Neville et al., 1997; Palmeri and Malim, 1999). NLS sequences within Rev enable it to shuttle back to the nucleus, where it again interacts with importin-β to be transported through the nuclear pores into the nucleus (Henderson and Percipalle, 1997). Once exported, the HIV mRNAs are translated and the structural proteins are targeted to the cell membrane for virion assembly, with the exception of the Vpu/Env mRNA, which is translated on the ribosomes of the rough ER (Fig. 1.7, step 9).
1.5.3 Virion assembly

Following translation, the Gag and Gag-Pol precursors are targeted to the cellular membrane by N-terminal myristate moieties on Gag (section 1.3.2). Once bound to the membrane, the C-terminal NC domain of Gag encapsidates the RNA dimer, bound at the 5’ dimer initiation signal (DIS), via an interaction with the RNA packaging signal. The gp120:gp41 complexes are transported to the cell surface from the ER, possibly to lipid rafts, where an interaction between the cytoplasmic domain of gp41 and MA allows Env to become incorporated into the assembling virions (Campbell, Crowe, and Mak, 2001). Envelope that is not incorporated into virions is rapidly internalised into endocytic vesicles (Freed and Martin, 2001). Gag-Gag interactions, via NC and CA, as well as NC-RNA interactions drive the alignment of Gag particles, which forces the membrane outwards to eventually form a sphere tethered to the cell membrane prior to budding (Fig. 1.7, step 10). In addition to the structural viral proteins, the accessory proteins Vpr, Nef and cellular proteins including cyclophilin A as well as tRNA\textsubscript{lys}, which is required to initiate reverse transcription upon HIV infection of a target cell, are incorporated into the assembling virions.

1.5.4 Virion budding and maturation

The final step of the virus life cycle is the ‘pinching off’ of the tethered virion and the subsequent maturation of virion proteins (Fig. 1.7, step 11). The precise mechanisms by which the tethered particles bud from the cell membrane remain unclear; however, the HIV ‘late’ domain (PTAP) of p6 is involved (section 1.3.2.2). Once budded, HIV particles undergo a PR-dependent Gag and Gag-Pol processing to produce morphologically defined infectious virions, with a dense conical-shaped core characteristic of a mature virus particle.

1.6 HIV receptors

1.6.1 CD4

Specific attachment between HIV and the target cell occurs via an interaction between the HIV envelope glycoprotein gp120 and the immunoglobulin-like receptor CD4, whose normal cellular function is the binding of MHC-II on antigen presenting cells (Dalgleish et al., 1984; Klatzmann et al., 1984b; Maddon et al., 1986). This binding occurs between the N-terminal domain of CD4 and regions within gp120, and induces conformational changes in gp120 resulting in binding to a second receptor, and membrane fusion (section 1.7).
1.6.2 HIV coreceptors

The inability of CD4 to render murine cells permissive to HIV infection, and the observation that virus isolates exhibited a distinct tropism for CD4⁺ cell populations indicated that a second receptor(s), or coreceptor, was required by HIV (section 1.2.1) (Asjo et al., 1986; Berger, Murphy, and Farber, 1999; Maddon et al., 1986). There are two major HIV coreceptors – CCR5 and CXCR4. The expression patterns of these coreceptors correlates with both the cellular tropism (M- versus T-tropism) and biological phenotypes (NSI versus SI) previously used to classify HIV. All NSI strains can exploit CCR5 for infection, and all SI use CXCR4, although the two are not mutually exclusive – many primary SI strains can efficiently use both CXCR4 and CCR5 (Simmons et al., 1996; Valentin et al., 1994; Zhang et al., 1996). It rapidly became clear that coreceptor use was a more accurate characteristic by which to define virus isolates, and this lead to the new classification of HIV as CCR5-tropic (R5), CXCR4-tropic (X4), or dual-tropic (R5X4)(Berger et al., 1998). The cellular expression patterns and chemokine ligand information of these receptors, as well as all other 7TM GPCRs able to support infection by HIV and SIV, are described in Table 1.4.

1.6.2.1 CXCR4

CXCR4 was identified as the coreceptor for T-cell line adapted strains of HIV-1 (T-tropic) by the screening of a human cDNA library for genes able to support fusion of murine cells with T-tropic Envs when expressed with CD4 (Feng et al., 1996). Although many HIV-1 and HIV-2 strains, as well as FIV, are able to use CXCR4 as a coreceptor, the majority of SIV isolates use it weakly if at all, despite the presence of distinct M- and T-tropic SIV strains (Chen, Z. et al., 1997; Edinger et al., 1997a; Willett et al., 1997). CXCR4 is expressed on lymphocytes, monocyte-derived cells and some brain cell populations, and has crucial roles in B-cell lymphopoiesis and organ vascularisation, neuron migration and CNS patterning (Table 1.4)(Lavi et al., 1997; Nagasawa et al., 1996; Tachibana et al., 1998; Zou et al., 1998). As CXCR4 is the sole receptor for its ligand stromal cell-derived factor-1 (SDF-1α), a lack of either SDF-1α or CXCR4 has fatal effects during development (Nagasawa et al., 1996; Zou et al., 1998).

There are several distinct functional domains within CXCR4, and their importance as determinants of coreceptor activity is both virus- and cell-type dependent (Brelot et al., 1997; McKnight et al., 1997; Picard et al., 1997b; Strizki et al., 1997).
<table>
<thead>
<tr>
<th>Chemokine receptor</th>
<th>Virus</th>
<th>Ligands</th>
<th>Cellular Distribution</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR1 HIV-2</td>
<td>RANTES, MIP-1α, MIP-5, MCP-2, MCP-3</td>
<td>T, M, DC</td>
<td>(McKnight et al., 1998)</td>
<td></td>
</tr>
<tr>
<td>CCR2b HIV-1, HIV-2, SIV</td>
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<td>M, T</td>
<td>(Doranz et al., 1996)</td>
<td></td>
</tr>
<tr>
<td>CCR3 HIV-1, HIV-2</td>
<td>Eotaxin, -2, -3, RANTES, MCP-2, -3, -4, MIP-5</td>
<td>E, MG, Th2</td>
<td>(Choe et al., 1996)</td>
<td></td>
</tr>
<tr>
<td>CCR4 HIV-2</td>
<td>TARC, MDC</td>
<td>Th2, Br</td>
<td>(McKnight et al., 1998)</td>
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<tr>
<td>CCR5 HIV-1, HIV-2, SIV</td>
<td>RANTES, MIP-1α, MIP-1β, MCP-2</td>
<td>T, M, DC</td>
<td>(Deng et al., 1996)</td>
<td></td>
</tr>
<tr>
<td>CCR8 HIV-1, HIV-2, SIV</td>
<td>L-309</td>
<td>Th2, Br</td>
<td>(Rucker et al., 1997)</td>
<td></td>
</tr>
<tr>
<td>CCR9 HIV-1</td>
<td>CC chemokines</td>
<td>L, B, placenta, thymus</td>
<td>(Choe et al., 1998a)</td>
<td></td>
</tr>
<tr>
<td>CXCR2 HIV-2</td>
<td>IL-8, GRO-α, -β, -γ</td>
<td>N, Br</td>
<td>(Bron et al., 1997)</td>
<td></td>
</tr>
<tr>
<td>CXCR4 HIV-1, HIV-2, SIV</td>
<td>SDF-1α</td>
<td>L, M, Br, P</td>
<td>(Feng et al., 1996)</td>
<td></td>
</tr>
<tr>
<td>CXCR5 HIV-2</td>
<td>BCA-1</td>
<td>B cells</td>
<td>(Kanbe et al., 1999)</td>
<td></td>
</tr>
<tr>
<td>CXCR6 HIV-1, HIV-2, SIV</td>
<td>CXCL16</td>
<td>T, M, placenta</td>
<td>(Deng et al., 1997)</td>
<td></td>
</tr>
<tr>
<td>CX3CR1 HIV-1, HIV-2</td>
<td>Fractalkine</td>
<td>NK, Th1, Br</td>
<td>(Reeves et al., 1997; Rucker et al., 1997)</td>
<td></td>
</tr>
<tr>
<td>GPR1 HIV-1, HIV-2, SIV</td>
<td>Unknown</td>
<td>Tissue M, Br</td>
<td>(Farzan et al., 1997a)</td>
<td></td>
</tr>
<tr>
<td>GPR15 HIV-1, HIV-2, SIV</td>
<td>Unknown</td>
<td>T, colon</td>
<td>(Deng et al., 1997; Farzan et al., 1997a)</td>
<td></td>
</tr>
<tr>
<td>APJ HIV-1, HIV-2, SIV</td>
<td>Apelin</td>
<td>CNS</td>
<td>(Choe et al., 1998a)</td>
<td></td>
</tr>
<tr>
<td>RDC1 HIV-1, HIV-2, SIV</td>
<td>Unknown</td>
<td>L, Br</td>
<td>(Shimizu et al., 2000)</td>
<td></td>
</tr>
<tr>
<td>US28 HIV-2</td>
<td>-</td>
<td>-</td>
<td>(Pleskoff et al., 1997)</td>
<td></td>
</tr>
<tr>
<td>LTB4 HIV-1</td>
<td>Leukotriene</td>
<td>-</td>
<td>(Owman et al., 1998)</td>
<td></td>
</tr>
</tbody>
</table>

Table 1.4 Human chemokine receptors and HIV coreceptors. A list of all known chemokine receptors and orphan 7-TM receptors known to support infection by HIV/SIV, including their natural ligands and cellular distribution. The main two HIV coreceptors, CCR5 and CXCR4, are highlighted. T = T-cells, M = monocytes, DC = dendritic cells, B = B-cells, E = eosinophils, MG = microglia, MØ = macrophage, L = lymphocytes, Br = brain, N = neutrophils, P = progenitor cells.
All extracellular regions of CXCR4, including the N-terminus and all three extracellular loops (ECL1, ECL2 and ECL3), have been implicated for its function as an HIV and FIV coreceptor, (Brelot et al., 1997; Doranz et al., 1999; Picard et al., 1997b; Willett et al., 1998; Zhou et al., 2001). In particular, tyrosine residues in the N-terminus and the second extracellular loop are proposed to be crucial for gp120 binding and support of infection, although some isolates remain unaffected by a deletion of the N-terminus (Kajumo et al., 2000; Picard et al., 1997b; Pontow and Ratner, 2001). In addition, negatively charged residues within ECL2 are proposed to form high affinity electrostatic interactions with proximal positively charged residues within the V3 loop of X4-utilising HIV strains (Bhattacharyya, Brooks, and Callahan, 1996; Kirchhoff et al., 1995). SDF-1α binds to the N-terminus of CXCR4, and mediates signalling effects via ECL2, and although both these regions are exploited by gp120, it is thought that intracellular signalling induced by HIV binding does not play a major role in HIV entry (Doranz et al., 1999; Potempa et al., 1997; Zhou et al., 2001). In addition to supporting infection of CD4+ cells by HIV/SIV, several strains can utilise CXCR4 in a CD4-independent manner (Clapham, McKnight, and Weiss, 1992; Endres et al., 1996; Hoxie et al., 1998; Liu, H. et al., 2000; Reeves et al., 1999).

1.6.2.2 CCR5

The identification of CXCR4 as an HIV coreceptor, in conjunction with early observations that β-chemokines such as RANTES, MIP-1α and MIP-1β inhibit HIV infection, led to the discovery of CCR5 as the second coreceptor for HIV (Cocchi et al., 1995; Deng et al., 1996; Dragic et al., 1996; Oravecz, Pall, and Norcross, 1996). This chemokine receptor is expressed on T-cells, macrophages and dendritic cells, and interacts with β-chemokines to induce chemotaxis of immune cells to sites of infection and inflammation (Table 1.4)(Deng et al., 1996). Although CCR5 is predominantly exploited by M-tropic isolates of HIV-1, most primary SI strains are dual-tropic and thus able to exploit both CCR5 in addition to CXCR4 (section 1.6.2.5). The interaction of gp120 with CD4 is proposed to increase its binding affinity for CCR5, and thus the use of CCR5 in a CD4-independent manner has been observed, but is rare (Edinger et al., 1997b; Hill et al., 1997; Reeves et al., 1999; Wu et al., 1996).

The importance of CCR5 in HIV transmission is highlighted by the observation that individuals homozygous for a 32-base pair deletion in CCR5 (Δ32/Δ32) are largely
resistant to HIV (Dean et al., 1996; Samson et al., 1996). This defect removes three of the transmembrane (TM) domains and two of the extracellular loops (ECLs), and thus prevents cell surface expression of the mutant protein. Some individuals possessing this genotype have become infected with HIV, and they predominantly harbour X4-using isolates, although one individual carries variants able to efficiently use CCR5 (Biti et al., 1997; Gorry et al., 2002b; Liu et al., 1996; Michael et al., 1998; Naif et al., 2002; O'Brien et al., 1997; Theodorou et al., 1997). Those heterozygous for this Δ32 polymorphism are not protected from infection, yet do demonstrate a slower rate of disease progression, possibly due to lower cell surface expression levels of CCR5 (Huang et al., 1996; Stewart et al., 1997; Wu et al., 1997b)). It has been proposed that several CCR5 molecules are required to form an entry complex functional for HIV coreceptor activity (Kuhmann et al., 2000). As such, low cell surface concentrations of CCR5 would render these complexes less able to form, and thus reduce sensitivity to infection. In addition to the Δ32 deletion, CCR5 displays other polymorphisms affecting HIV pathogenesis and disease progression, including several in the promoter region (Martin et al., 1998). Homozygosity for two common polymorphisms in the CCR5 promoter region (CCR5 59029A and CCR5 59353C) appears less frequently in LTNPs, implying these regions have negative effects on HIV disease progression (Clegg et al., 2000).

Like CXCR4, CCR5 can be split into several functionally distinct domains, including regions important for chemokine binding (ECL2), for intracellular signaling (conserved DRY motif in the second intracellular loop), and important for HIV coreceptor function (Fig. 1.9) (Doranz et al., 1997b; Lee et al., 1999; Rucker et al., 1996; Samson et al., 1997; Zhou et al., 2000). Although there are regions in CCR5 necessary for gp120-binding and thus coreceptor function, including the N-terminus, in particular, sulfated tyrosine residues in this location, and residues (such as G163) at the TM4/ECL2 junction, there is some degree of virus isolate dependence and a general requirement for the structural integrity of the extracellular domains (Bieniasz et al., 1997; Choe et al., 1998b; Dragic et al., 1998; Farzan et al., 1998; Farzan et al., 1999; Genoud et al., 1999; Hill et al., 1998; Picard et al., 1997a; Siciliano et al., 1999). Whilst remaining functionally distinct, there is some overlap between the gp120-, and ligand-binding domains of CCR5 (Alkhatib et al., 1997a; Lee et al., 1999; Olson et al., 1999). It has been demonstrated that intracellular signaling occurs in response to gp120
binding to CCR5 (Arthos et al., 2000; Davis et al., 1997; Liu, Q. et al., 2000; Oppermann et al., 1999). However, this signaling effect is not required for either the coreceptor function of CCR5, and the role of such signaling in HIV pathogenesis is currently unclear (Alkhatib et al., 1997b; Amara et al., 2003; Aramori et al., 1997; Farzan et al., 1997b; Popik and Pitha, 1998; Weissman et al., 1997).

![Diagram of CCR5 functional domains](image)

**Figure 1.9. Functional domains of CCR5.** A diagrammatic representation of CCR5, showing functional domains of CCR5 and important residues. Red lines represent putative disulfide bonds. Unlinked red lines represent the bond formed between the N-terminus and ECL3 when the receptor is circularised in the membrane. Green circles = residues important for intracellular signalling, yellow circles = residues important for ligand binding, dark grey circles = residues important for coreceptor activity. Tyrosine residues are lilac. Residues important for both chemokine and gp120 binding are yellow with a grey outline; tyrosine residues crucial for coreceptor activity are lilac with a grey outline.

1.6.2.3 Alternative 7TM coreceptors

In addition to CCR5 and CXCR4, many other chemokine receptors and related 7TM GPCRs can be exploited by HIV/SIV to infect CD4+ indicator cell lines *in vitro*. As seen with CCR5 and CXCR4, the V1/V2 and V3 loops of gp120 determine the capacity of HIV to exploit these alternative coreceptors (section 1.7.3)(Hoffman et al., 1998). The expansion in coreceptor use of HIV-1 from R5 to R5X4 or X4 during the later stages of disease often confers the capacity to use alternative coreceptors in *vitro*; however, HIV-2 and SIV strains are potentially more promiscuous in this regard (section 1.6.2.5 and Chapter 4)(Clapham and McKnight, 2002; Doms, Edinger, and
Moore, 1998; McKnight et al., 1998; Morner et al., 1999; Reeves and Doms, 2002). Some of these coreceptors, e.g. CCR8 and CXCR6, can be used by patient isolates to infect primary lymphocytes (Lee et al., 2000; Sharron et al., 2000; Zhang, Y. et al., 1998b); however, their role in the majority of in vivo infections and HIV pathogenesis is currently thought to be insignificant (Table 1.4 and Chapter 4)(http://hiv-web.lanl.gov)(Clapham and Weiss, 1997; Willey et al., 2003a; Willey et al., 2003b). Yet, the importance of such alternative coreceptors, particularly in the advent of coreceptor-specific HIV therapeutics, cannot be underestimated.

1.6.2.4 Virally-encoded chemokine receptors

Many viruses have evolved to exploit and modulate the host immune system to their advantage by encoding and synthesising chemokines and chemokine receptors (Fernandez and Lolis, 2001; Fernandez and Lolis, 2002; Lalani and McFadden, 1999; Lusso, 2000; McFadden et al., 1998; Moore et al., 1996; Pease and Murphy, 1998; Rosenkilde et al., 2001; Stine, Chantry, and Gray, 1999; Wells and Schwartz, 1997). For example, the Shope Fibroma virus, myxoma virus, cowpox virus, variola virus and ectromelia all synthesise cytokine/chemokine receptors or receptor analogues (Alcamí et al., 1999; Alcamí and Smith, 1996; Fernandez and Lolis, 2002; Kotwal, 2000; McFadden et al., 1998; Nash et al., 1999; Smith, Bryant, and Alcamí, 2000). Many members of the herpesvirus genus, including HHV6, HHV8 and herpesvirus Saimiri also encode chemokine receptors or GPCRs (Cesarman et al., 1996; Dockrell, 2003; Guo et al., 1997; Isegawa et al., 1998; Milne et al., 2000; Rosenkilde et al., 2001). In addition to immunomodulatory functions, some viral chemokine receptor analogues have other functions. For example, one of the three chemokine receptor analogues encoded by CMV, US28, supports infection by several HIV and SIV isolates (Table 1.5)(Gao and Murphy, 1994; Kuhn, Beall, and Kolattukudy, 1995; Pleskoff et al., 1997; Rosenkilde et al., 2001; Rucker et al., 1997).

1.6.2.5 Evolution of coreceptor use in vivo

With the exception of infected Δ32/Δ32 individuals, most transmitted viruses and those present in primary infection are CCR5-using and of a non-syncytium-inducing (NSI) phenotype, and such strains persist throughout disease, even in the presence of antiretroviral therapy (Schuitemaker et al., 1991; van Rij et al., 2002; van't Wout et al., 1994; Zhu et al., 1993). Syncytium-inducing (SI) isolates able to exploit CXCR4 for infection in addition to, or instead of, CCR5 (X4 or R5X4) only emerge
during the later stages of disease in approximately 50% of HIV\(^+\) individuals (Tersmette \textit{et al.}, 1988). This switch occurs in most HIV-1 subtypes, although X4 strains are rare in subtype C, and is associated with sequence changes within the coreceptor-determining V3 loop of gp120, as well as an increased rate of CD4\(^+\) T-cell decline and progression to AIDS (section 1.7.3 and Chapter 5)(Bjorndal \textit{et al.}, 1999; Cecilia \textit{et al.}, 2000; Connor \textit{et al.}, 1997; Fouchier \textit{et al.}, 1996; Jansson \textit{et al.}, 1996; Peeters \textit{et al.}, 1999; Richman and Bozzette, 1994; Schuitemaker \textit{et al.}, 1992). The expansion to use CXCR4 as well as CCR5 is often associated with the use of alternative coreceptors \textit{in vitro}, and may enable HIV to target new CD4\(^+\) cell populations, such as naïve CD4\(^+\)CXCR4\(^+\) T-cells. It is possible that the increased cytopathicity observed upon this coreceptor switch is mediated via gp120 interaction with and apoptosis induction of CD4\(^+\)CXCR4\(^+\) bystander cells (Jekle \textit{et al.}, 2003). However, as all HIV\(^+\) patients eventually progress to AIDS in the absence of R5X4/X4 strains, other host and/or viral factors play a role (de Roda Husman \textit{et al.}, 1999).

The explanation for preferential transmission of R5 viruses and subsequent evolution of R5X4/X4 isolates is uncertain, but the existence of tissue compartments with specific cytokine and/or cellular microenvironments may favour the evolution of specific viral phenotypes. For example, the CXCR4 ligand and inhibitor of T-tropic isolates, SDF-1\(\alpha\), is constitutively expressed at mucosal surfaces, which may prevent transmitted X4 virus from establishing infection (Agace \textit{et al.}, 2000). In addition, FDCs upregulate CXCR4 expression on CD4\(^+\) T-cells within germinal centres following cell-cell contact, and lymphoid tissues, the major source of SDF-1\(\alpha\), are progressively destroyed during HIV infection (Estes \textit{et al.}, 2002; Pantaleo \textit{et al.}, 1993). The removal of this source of an inhibitory chemokine may alter selective pressures that prevent the establishment of a CXCR4-tropic viral quasispecies. A final point is the switch in cytokine profiles, and associated changes in coreceptor expression patterns, from type 1 to type 2 as disease progresses (section 1.2.1.1). However, why this phenotypic switch is not a common feature in all patients and across all HIV genetic subtypes is unclear.

\textbf{1.6.3 Chemokines}

\textit{1.6.3.1 Structure and function of chemokines}

Chemokines are a family of small chemotactic cytokines, subdivided according to the arrangement of the first two cysteine residues. These cysteines form disulfide bonds that stabilise the N-terminus (C1 and C3), and downstream loops (C2 and C4)
connecting three β-strands of the tertiary core structure (Fernandez and Lolis, 2002). Chemokines with a single amino acid separating the cysteine residues are termed CXC, or α-chemokines; those with no intervening residues are CC, or β-chemokines, and those with three residues separating the cysteines (of which there is only one defined - CX3C, or fractalkine) are γ-chemokines. The δ-chemokine, lymphotactin, is the only exception to this chemokine sequential rule-of-thumb, as it only possesses the first of the two cysteines (Kelner et al., 1994).

Chemokines are either homeostatic (constitutively produced and secreted) or inflammatory (released from cells in response to infection), and exert their effects by binding to 7TM GPCRs. Some chemokines are promiscuous in their use of chemokine receptors, for example RANTES (binds CCR1, CCR5, and CCR9), whereas others, such as SDF-1α, are specific for a single receptor (CXCR4)(Table 1.4). As such, promiscuous chemokines are able to adapt and function normally in the absence of one of their receptors, but the removal of a receptor with only a single ligand can be fatal. This is exemplified by Δ32/Δ32 CCR5 individuals who remain healthy despite a non-functional protein, and in the CXCR4 knock-out mouse system, where offspring rarely survive beyond birth (Dean et al., 1996; Nagasawa et al., 1996; Samson et al., 1996).

1.6.3.2 Virally-encoded chemokines

In addition to encoding chemokine receptor homologues, many pathogenic organisms synthesise and secrete chemokine homologues that act as agonists or antagonists, and manipulate the host immune response in their favour (Fernandez and Lolis, 2001; Fernandez and Lolis, 2002; Lalani and McFadden, 1999; Lusso, 2000; McFadden et al., 1998; Moore et al., 1996; Pease and Murphy, 1998; Rosenkilde et al., 2001; Stine, Chantry, and Gray, 1999; Wells and Schwartz, 1997). The poxvirus MCV (molluscum contagiosum virus) secretes an N-terminally truncated CC chemokine MC148, which exerts antagonistic properties on human CCR8 and is proposed to prevent the induction of virus-specific inflammatory responses (Damon, Murphy, and Moss, 1998; Luttichau, Gerstoft, and Schwartz, 2001; Luttichau et al., 2001). Other poxviruses including Ectromelia virus, Vaccinia virus, cowpox virus and smallpox, secrete a chemokine-binding protein (e.g. vCKBP, or vCC1 in Vaccinia, and M-T1 in myxoma virus), which exerts its effects by directly binding to the chemokine as opposed to the chemokine receptor (Fernandez and Lolis, 2002; Kotwal, 2000; Smith, Bryant,
and Alcami, 2000). The vCCl protein secreted by Vaccinia virus binds to a large number of chemokines, including MCP-1, and M-T1 binds to cell surface GAGs as well as MCP-1 (Alcami et al., 1998; Burns et al., 2002; Seet et al., 2001a; Seet et al., 2001b; Smith, 1999).

The human herpesviruses CMV, HHV-6 and HHV-8 encode a range of chemokine homologues (Dockrell, 2003; Zou et al., 1999). Human herpesvirus 8 (HHV-8), or Kaposi's sarcoma herpesvirus (KSHV), encodes three chemokines – vMIP-I, vMIP-II and vMIP-III (Nicholas et al., 1997). Although vMIP-I and vMIP-II are 48% identical at the amino acid level (Boshoff et al., 1997) their chemokine receptor-binding profiles differ extensively. vMIP-I is an agonist of CCR8, a potential agonist of GPR1 and antagonist of CXCL16 (Dairaghi et al., 1999; Endres et al., 1999; Haque et al., 2001; Simmons et al., 2000). In contrast, vMIP-II is an antagonist of CCR1, CCR2, CCR5, CCR10, CXCR4, CX3CR1 and XCR1, and an agonist of CCR3 and CCR8 (Boshoff et al., 1997; Endres et al., 1999; Fernandez and Lolis, 2001; Kledal et al., 1997; Luttichau et al., 2001; Shan et al., 2000). The third chemokine encoded by KSHV, vMIP-III, is more distantly related to both vMIP-I and vMIP-II (approximately 37% sequence similarity) as well as human chemokines (35% and 38% identity to TARC and eotaxin respectively), and it is primarily an agonist for CCR4, although it does weakly bind CCR8, GPR1 and CXCR6 (Dairaghi et al., 1999; Simmons et al., 2000; Stine et al., 2000).

1.6.3.3 Inhibition of HIV by chemokines and chemokine analogues

Prior to the discovery that chemokine receptors were the second receptors for HIV, it was known that chemokines inhibit HIV replication (Cocchi et al., 1995). Subsequent studies demonstrated that β-chemokines able to bind CCR5, like RANTES and, to a lesser extent MIP-1α and MIP-1β, inhibited HIV infection, but those unable to interact with CCR5 (MCP-1, MCP-2, MCP-3 and eotaxin) did not (Deng et al., 1996; Dragic et al., 1996; Oravecz, Pall, and Norcross, 1996). Other chemokine ligands able to inhibit HIV infection via their receptors include SDF-1α (CXCR4-mediated infection), eotaxin (CCR3), and I-309 (CCR8) (Bleul et al., 1996; Choe et al., 1996; Horuk et al., 1998; Oberlin et al., 1996; Virelizier, 1999). Not all coreceptors able to support HIV infection are chemokine receptors, but the ligands of such alternative coreceptors can still inhibit infection in the manner of chemokines. For example apelin
blocks infection through the 7TM GPCR APJ (Cayabyab et al., 2000; Zou et al., 2000). The two main mechanisms of chemokine-mediated HIV inhibition are (1) a steric block of gp120:chemokine receptor binding, and (2) an induction of receptor internalisation as seen with CCR5, CXCR4 and APJ (Alkhatib et al., 1997b; Amara et al., 1997; Arenzana-Seisdedos et al., 1996; Mueller, Kelly, and Strange, 2002; Signoret et al., 1997; Simmons et al., 1997; Zhou et al., 2003). The sensitivity of a range of cell types to β-chemokine-mediated inhibition of HIV is dependent in part upon the efficiency of CCR5 internalisation (Brandt et al., 2002).

Although chemokines are effective at inhibiting infection by all genetic subtypes of HIV (Trkola et al., 1998), their use as a therapeutic tool to suppress viral replication in vivo is not viable. In the first instance, elevated levels of chemokines could result in unwanted and prolonged inflammatory responses, although phase I and II clinical trials with high concentrations of MIP-1α as a cancer therapy did not cause adverse effects (Clemons et al., 1998; Marshall et al., 1998). A second consideration is that high concentrations of receptor-specific β-chemokines may force the evolution of HIV to use alternative coreceptors with greater efficiency (Virelizier, 1999). In addition, β-chemokines are relatively inefficient inhibitors of macrophage infection, an important viral reservoir (Arenzana-Seisdedos et al., 1996; Moriuchi et al., 1996; Simmons et al., 1997). As a final point, the expense of producing large quantities of a recombinant protein, and the requirement for intravenous administration, makes chemokines unsuitable as therapeutic agents.

Antagonistic chemokine analogues may be a suitable therapeutic alternative to chemokines (Simmons et al., 2000). N-terminal sequence differences between the two non-allelic isoforms of MIP-1α (LD78α and LD78β), and the resulting differences in chemotactic and HIV inhibitory properties, have demonstrated the importance of this region in chemokine activity (Aquaro et al., 2001; Menten et al., 1999; Nibbs et al., 1999; Proudfoot et al., 1999; Simmons et al., 1997). Subsequent studies of N-terminal modifications of RANTES found analogues with varying effects on RANTES receptors (Elsner et al., 2000; Proudfoot et al., 1999). The analogue MET-RANTES is an antagonist but is relatively inefficient at inhibiting infection by HIV, since the induction of CCR5 internalisation with this analogue is minimal (Elsner et al., 2001; Mack et al., 1998; Proudfoot et al., 1999; Simmons et al., 1997). However, the addition of an N-
terminal aminooxypentane group produces AOP-RANTES, a molecule that retains agonist activity against both CCR5 and CCR3, and demonstrates potent inhibition of CCR5-mediated HIV infection of both lymphocytes and macrophages (Simmons et al., 1997). The high binding affinity of AOP-RANTES for CCR5 induces a rapid internalisation of CCR5. This high binding affinity also prevents ligand dissociation in the low pH endosomes and thus prevents CCR5 recycling back to the cell surface (Mack et al., 1998).

Like AOP-RANTES, the analogue N°-nonanoyl-RANTES[2-68] (NNY-RANTES) is a potent inhibitor of HIV infection (Mosier et al., 1999). The agonistic/antagonistic properties of NNY-RANTES remain to be determined, but it has been shown to inhibit PBMC infection, induce CCR5 down-regulation and prevent CCR5 recycling more efficiently than even AOP-RANTES (Mosier et al., 1999; Sabbe et al., 2001). Preliminary in vivo studies with the SCID-huPBL mouse model show that although NNY-RANTES is a potent HIV inhibitor, some resistance, as well as escape to exploit CXCR4, was observed; this was, however, abrogated upon treatment in conjunction with AMD3100 (LaBranche et al., 2001; Mosier et al., 1999). Such modified chemokines removing cell surface CCR5 are promising candidates for anti-HIV therapy; however, the separation of activation and HIV-inhibitory functions of such molecules, to produce a full antagonist that retains potent inhibitory effects, is required. As with all coreceptor-specific inhibitors, the evolution of viral resistance remains a concern.

In addition to chemically modified chemokines, the viral chemokine analogues encoded and synthesised by KSHV are able to inhibit infection by HIV. vMIP-I blocks HIV-1 and -2 infection through several alternative coreceptors, including CCR8, GPR1, and, to a lesser extent CXCR6 and CCR3 (Kledal et al., 1997; Moore et al., 1996; Simmons et al., 2000; Willey et al., 2003a). The efficiency of inhibition through these coreceptors correlates with its agonistic or antagonistic properties. vMIP-I is a selective agonist of CCR8 and therefore an efficient inhibitor of CCR8-mediated infection (Chapter 4)(Dairaghi et al., 1999; Haque et al., 2001). The much broader chemokine receptor-binding repertoire of vMIP-II results in it being able to block HIV infection via CCR5 and CXCR4, albeit less potently than their natural ligands RANTES and SDF-1α (Boshoff et al., 1997; Kledal et al., 1997). vMIP-II has high affinity for CCR3 and is a potent CCR3 agonist. As such, CCR3-mediated infection is inhibited more efficiently
by vMIP-II than by its natural ligand eotaxin (Kledal et al., 1997). The third KSHV chemokine, vMIP-III, is a much less efficient inhibitor, only weakly blocking HIV infection via CCR8 and GPR1 (Simmons et al., 2000; Stine et al., 2000).

1.6.3.4 Enhancement of HIV infection by chemokines

It has been extensively demonstrated that chemokines inhibit infection by HIV at low concentrations, but there have been many observations that the β-chemokine RANTES enhances infection at high concentrations (Gordon et al., 1999). This enhancement occurs by two mechanisms. Firstly, RANTES increases virus-cell adsorption, as at high concentrations it oligomerises and cross-links GAGs on both the cellular and the viral membrane (Hoogewerf et al., 1997; Kuschert et al., 1999; Proudfoot et al., 2001; Trkola et al., 1999). Oligomerisation of RANTES is necessary for enhancement, as RANTES mutants unable to oligomerise, or MIP-1α and MIP-1β, which do not form multimers, do not enhance (Czaplewski et al., 1999). RANTES-mediated enhancement of infection is not CCR5-specific, or fusion-dependent, as it also enhances infection by X4 HIV isolates and MuLV and VSV pseudotyped HIV particles (Gordon et al., 1999; Trkola et al., 1999). SDF-1α, the ligand for CXCR4, binds heparan sulphates, but this appears to enhance its anti-HIV effects rather than enhancing infection (Valenzuela-Femandez et al., 2001).

The second enhancement mechanism involves intracellular signal transduction pathways. At low concentrations (1-100 nm), RANTES binds to GPCRs and stimulates a transient increase in intracellular calcium levels. Higher concentrations of RANTES (1 μM) induce a prolonged increase in [Ca^{2+}], via interactions with cell surface GAGs (Bacon et al., 1995; Chang et al., 2002; Gordon et al., 1999; Trkola et al., 1999). This second signalling response activates phosphotyrosine kinase (PTK) and mitogen-activated protein kinase (MAPK) dependent pathways which may enhance virus infectivity by activating cellular transcription factors or by directly phosphorylating viral proteins. The physiological relevance of such enhancement is not clear. Plasma concentrations of RANTES rarely exceed 25 nM, yet local concentrations at sites of inflammation are likely to be elevated, and the sequestration of RANTES to lymphoid tissues by GAGs may increase these levels further (Kakkanaiah, Ojo-Amaize, and Peter, 1998; Krowka et al., 1997; McKenzie et al., 1996; Muller et al., 1999). The implication of this in HIV pathogenesis is not clear.
1.7 HIV entry

Like other viruses and pathogenic organisms, HIV entry occurs following an interaction between the envelope protein and cellular receptors. In the case of influenza, the envelope protein haemagglutinin binds to sialic acid residues on glycoproteins and glycolipids. The malarial-causing *Plasmodium vivax* interacts with the Duffy antigen receptor complex (DARC), and with HIV, the envelope glycoprotein gp120 interacts with CD4 and a chemokine receptor. Unlike other viruses, such as influenza which require a low pH environment to trigger fusion, HIV entry is pH-independent, with gp41-mediated membrane fusion events being triggered by receptor binding (McClure, Marsh, and Weiss, 1988; Stein et al., 1987). HIV entry may occur at the cholesterol-rich membrane microdomains known as lipid rafts, as CD4 and the chemokine receptors CCR5 and CXCR4 may colocalise in these regions; however, the importance of these domains in HIV entry remains controversial (Manes et al., 2000; Percherancier et al., 2003; Popik, Alce, and Au, 2002; Singer et al., 2001; Xiao et al., 1999).

1.7.1 Envelope structure

The cell surface subunit of HIV, gp120, possesses a complex structure. It is composed of five highly conserved and five hypervariable regions, termed C1-C5, and V1-V5, respectively (Fig. 1.10A) (Alizon et al., 1986; Modrow et al., 1987; Starcich et al., 1986; Willey et al., 1986). The five variable loops of gp120 are exposed on the extracellular surface, and thus are immunogenic epitopes; however, the high degree of variation in these regions both within and between individuals results in any induced neutralising antibodies becoming ineffective relatively quickly (section 1.2.4.2) (Modrow et al., 1987). In addition to the conserved regions, the 18 cysteine residues located within gp120 are highly conserved, and these form nine intramolecular disulfide bonds, four of which anchor the bases of the variable loops V1-V4 (Leonard et al., 1990).

1.7.2 Interaction with CD4 and conformational changes

The complementarity-determining region (CDR2) located within the N-terminal domain of CD4, D1, binds with high-affinity to a cleft in gp120 situated between the inner and outer domains of gp120, and adjacent to the ‘bridging sheet’, a series of four anti-parallel β-sheets situated in between the inner and outer domains of the gp120 core.
Figure 1.10 Gp120 structure and Gp120:CD4 interaction. Organisation of the variable loops and constant domains of gp120 (A). The X-ray crystallographic structure of the HIV-1 gp120 core domain (B) and a gp120:CD4 complex (C) is shown, with the inner and outer domains of gp120, as well as the bridging sheet, the CD4-binding hydrophobic cleft and the coreceptor binding sites highlighted. A diagrammatical representation of gp120 bound to CD4, with residues important for this interaction highlighted in both molecules (D). Panel (A) adapted from (Hoffman and Doms, 1999). Panels (B), (C) and (D) adapted from (Kwong et al., 1998).
Specific amino acids within both CD4 and gp120 have been implicated in this interaction. F43 (phenylalalanine) in D1 of CD4, and positively-charged residues in CDR2 spatially proximal to F43 [K46 (lysine) and R59 (arginine)] make multiple contacts with gp120, and possibly assist interactions by stabilising the hinge region of CD4 (Fig. 1.10B) (Choe and Sodroski, 1992; Kwong et al., 1998; Moebius et al., 1992; Murray et al., 2002). In particular, they are proposed to interact with residues within the fourth conserved region [D368 and D457 (aspartic acid)] and within the V1/V2 loop stem, or bridging sheet, of gp120 [W427 (tryptophan) and L125 (leucine)], which line the hydrophobic cavity filled by F43 and located in between the inner and outer domains of gp120 (Fig. 1.10A and 1.10C) (Kwong et al., 1998; Olshevsky et al., 1990; Wyatt et al., 1992). Thermodynamic analysis, and the fact that binding of CD4 induces the exposure of neutralising antibody epitopes on gp120, indicates that extensive conformational changes occur upon gp120 and CD4 interactions (section 1.2.4.2) (Kang et al., 1993; Myszka et al., 2000; Sattentau and Moore, 1991; Thali et al., 1993). Such conformational changes upon CD4-binding include a shifting of the V1/V2 and the V3 loops (Sullivan et al., 1998; Wyatt et al., 1995).

1.7.3 Coreceptor interaction and conformational changes

Several regions of gp120 are important for coreceptor binding, and thus HIV entry (Hoffman and Doms, 1999). Even prior to the discovery of HIV coreceptors, it was observed that regions outside of the CD4-binding site were important for the cellular tropism of HIV and the binding of HIV to CD4 (Liu et al., 1990; O'Brien et al., 1990; Shioda, Levy, and Cheng-Mayer, 1991). In particular, the V3 loop is a major determinant of tropism and biological phenotype, with sequences in the V1/V2 loops, the V4 and V5 loops and C4 domain playing a minor role (Boyd et al., 1993; Chesebro et al., 1992; Cho et al., 1998; De Jong et al., 1992; Fouchier et al., 1992; Groenink et al., 1993; Hwang et al., 1991; Labrosse et al., 2001; Shioda, Levy, and Cheng-Mayer, 1992; Simmons et al., 1995; Singh and Collman, 2000; Takeuchi et al., 1991).

The V1/V2, V4, V5 and C4 regions indirectly influence coreceptor binding and viral phenotype due to their proximity to, or involvement in the exposure of, the coreceptor binding site (Hoffman et al., 2002). Binding of gp120 to CD4 (Fig. 1.11A) induces conformational changes that include a repositioning of the V1/V2 stem-loop, the V4 loop and the C4 domain, regions containing the discontinuous sequence recognised by CD4-induced (CD4i) epitopes recognized by neutralising mabs such as
17b and 48d (Thali et al., 1993; Wyatt et al., 1995; Wyatt et al., 1992). The displacement of gp120 domains, possibly by a reduction of gp120 disulfide bonds via cell surface protein-disulfide isomerases, exposes or creates a coreceptor binding site (Fig. 1.12B)(Barbouche et al., 2003; Rizzuto et al., 1998; Trkola et al., 1996a; Wu et al., 1996). Gp120 subsequently binds the coreceptor (Fig. 1.11C), at which point gp120 is lost and gp41-mediated membrane fusion is initiated (Fig. 1.11D)(section 1.7.4).

Figure 1.11. Gp120:coreceptor interaction. Diagrammatical representation of gp120:receptor interactions. The trimeric gp120 spike interacts with CD4 (A), which induces conformational changes in gp120 to create/expose the coreceptor-binding site (B). Following binding to the coreceptor (C), gp120 is shed and gp41-mediated membrane fusion events ensue (D).

Specific determinants for coreceptor binding, as well as post-binding coreceptor interactions, are found within the V3 loop (Choe et al., 1996; Cocchi et al., 1996; Platt et al., 2001; Speck et al., 1997). The stem of the V3 loop and the C4 domain contain positively-charged residues which directly interact with the N-terminal domain of CCR5 in vitro, whereas sequences in the V3 crown, including the GPGX V3 tip sequence, interact with extracellular regions (likely ECL2) of CCR5/CXCR4, possibly by
mimicking the β-hairpin structures of the natural chemokine ligands (Cormier and Dragic, 2002; Cormier et al., 2001; Hung, Vander Heyden, and Ratner, 1999; Isaka et al., 1999; Sharon et al., 2003; Shimizu et al., 1999; Verrier et al., 1999; Wyatt et al., 1995). The presence of uncharged residues (serine/glycine) at position 11 within the V3 loop, and of negatively-charged residues (aspartic/glutamic acid) at position 25, are strong indicators of CCR5-use and a NSI phenotype, and correspondingly, V3 loops with a higher overall positive charge are associated with X4-use and a SI phenotype (Hung, Vander Heyden, and Ratner, 1999; Xiao et al., 1998a). In addition to the V3 loop, the β19 strand of gp120, which forms part of the bridging sheet, is important for CXCR4 binding (Basmaciogullari et al., 2002). Thus, the gp120/CD4/coreceptor interactions are complex, and although many critical features have been identified, there remains a large degree of strain-specific variation rendering characterisation of this entry pathway incomplete.

1.7.4 Gp41-mediated membrane fusion

Early studies investigating HIV-induced syncytium formation demonstrated the importance of the envelope protein for cell fusion (Lifson et al., 1986; Sodroski et al., 1986). In fact it is the transmembrane subunit of the envelope glycoprotein, gp41, which is responsible for virus:cell fusion events. Like other viruses, including influenza, Newcastle disease virus and Ebola, the N-terminus of HIV/SIV gp41/32 is composed of a series of hydrophobic residues which form the ‘fusion peptide’ and as such, are crucial for HIV infectivity (Bosch et al., 1989; Cao et al., 1993; Freed, Myers, and Risser, 1990; Gallaher, 1987). The importance of this region for HIV infectivity is highlighted by the observation that short peptide sequences mimicking this peptide are highly effective fusion inhibitors (section 1.8.3.2)(Slepushkin et al., 1993). In addition to the N-terminal fusion peptide, gp41 contains other structural motifs important for fusion induction, including an N-terminal heptad repeat leucine zipper-like helical coiled coil, conserved within HIV and other virus families, and a C-terminal amphipathic helical domain (Fig. 1.12A)(Chambers, Pringle, and Easton, 1990; Delwart, Mosialos, and Gilmore, 1990; Fujii et al., 1992; Gallaher et al., 1989; Slepushkin et al., 1992; Weissenhorn et al., 1998). The N-terminal helix is involved in gp120 attachment as well as fusion (Chen, 1994).
Figure 1.12. Gp41 structure and mechanism of membrane fusion. The transmembrane subunit of the HIV envelope, gp41, possesses distinct functional domains, including a hydrophobic fusion peptide, a N-terminal leucine zipper-like helix, a C-terminal amphipathic helix and a transmembrane portion (A). Binding of gp120 to CD4 and a coreceptor induces conformational changes within gp41, resulting in insertion of the fusion peptide into the target cell membrane (B). The N-terminal region then snaps back, with the N- and C-terminal trimeric helices forming a six-helix bundle (C). Several of these act together to form a fusion pore, which gradually pulls the membranes closer together to allow mixing (D) and subsequent fusion (E).
As well as inducing conformational changes in gp120, the interaction of gp120 with CD4 and the appropriate coreceptor initiates structural changes in gp41, proposed to reveal the fusion peptide and disengage gp120 (Fig. 1.11D)(Abrahamyan et al., 2003; Hart et al., 1991; Jones, Korte, and Blumenthal, 1998; Sattentau and Moore, 1991). As earlier characterised with influenza HA2, the fusion peptide then inserts into the cellular membrane (Fig. 1.12B), and the N- and C-terminal helices (which exist on the virion surface as trimers, like gp120) snap back to form a six-helix bundle parallel to the virus:cell membranes (Fig. 1.12C), with the N-helices forming a central core, and the C-terminal helices inserting into the external hydrophobic grooves (Brasseur et al., 1988; Carr and Kim, 1993; Chan et al., 1997; Chen et al., 1995; Stegmann et al., 1991; Weissenhorn et al., 1997; Wild et al., 1994). Several of these gp41 trimers act together to form a fusion pore, following coreceptor binding yet prior to the formation of the six-helix bundle, which gradually pulls the cellular and viral membranes close enough to overcome thermodynamic restrictions and allow membrane mixing (Fig. 1.12D) and, consequently, fusion (Fig. 1.12E)(Abrahamyan et al., 2003; Golding et al., 2002; Markosyan, Cohen, and Melikyan, 2003; Weissenhorn et al., 1999).

1.7.5 CD4-independent infection

Binding of gp120 to CD4 induces conformational changes that reveal the coreceptor binding site. However, CD4-independent HIV and SIV isolates are found (Edinger et al., 1997b; Endres et al., 1996; Gorry et al., 2002a; Hesselgesser et al., 1997; Hoxie et al., 1998; Liu et al., 2000a; Reeves et al., 1999). The envelopes of such isolates exist in a state whereby the coreceptor binding site is already partially or fully exposed, and thus can be triggered to change structure with less activation energy (Hoffman et al., 1999; Reeves and Schulz, 1997). Residues in gp120 important for a CD4-independent phenotype include some at the base of the V4 loop and the C4 domain (N386K, L387F, K403R, and I423V), some in the V2 loop and V1/V2 stem of gp120, and some in the V3 loop (R298K)(Edwards et al., 2001; Hoffman et al., 1999; Kolchinsky et al., 1999; LaBranche et al., 1999; Reeves and Schulz, 1997). Many of these mutations lie close to the coreceptor binding domain, or result in the loss of N-linked glycosylation sites, and thus may shift the positions of the V1/V2 and/or V3 loops, or render them more flexible, and thus expose the coreceptor-binding site and confer CD4-independence.
1.8 HIV Prevention and Therapeutic Approaches

1.8.1 HIV vaccine strategies

Several features of HIV make the ultimate hope of developing an HIV vaccine a daunting challenge. The high rate of replication and mutation, and consequently the rapid evolution of HIV, in combination with integration of HIV into the host genome and persistence of long-lived latently infected cells suggest that long-term success of a therapeutic HIV vaccine may be uncertain (Gaschen et al., 2002; Heeney and Hahn, 2000; Letvin, 1998; Mwau and McMichael, 2003). The success and development of sterilising vaccines for HIV i.e. those which aim to prevent infection, still remains unlikely for several reasons. The induction of mucosal immunity is thought to be essential to the success of a sterilising vaccine, as the mucosae are the main sites of HIV transmission. However, the mechanisms by which mucosal immunity is induced, as well as the nature of the initial cellular target for HIV within the genital tract and the mechanism of viral dissemination into the lymph nodes, remain ill-defined and continue to be challenges in the development of a preventative HIV vaccine (Heeney and Hahn, 2000; Letvin, 1998; McMichael, Mwau, and Hanke, 2002).

Traditional vaccines e.g. polio and hepatitis B vaccines, clear incoming virus by inducing a neutralising antibody response. Unfortunately, although anti-HIV antibodies are produced early in infection, HIV rapidly escapes neutralisation (Baltimore, 2002; Letvin, Barouch, and Montefiori, 2002; McMichael, Mwau, and Hanke, 2002; Mwau and McMichael, 2003). The study of HIV+ rapid progressors, LTNPs, and ESNs have identified a low viral load, and a strong antibody/anti-HIV CTL response during initial viraemia as being vital for subsequent control of, or protection against, infection/disease (section 1.2.3). Although a CTL response does not prevent infection of cells, it does kill them upon production of virus antigen. An HIV vaccine should thus aim to provide sufficient control of infection during the early stages of disease to allow the evolution and maintenance of a strong memory immune response (Ho and Huang, 2002). Indeed, recent vaccine developments are largely aimed at stimulating CTL responses, to reduce viral setpoint levels and thus give the host the potential to clear the virus (Cohen, 2002b; Letvin, 1998; McMichael, Mwau, and Hanke, 2002; Nabel, 2002). The classic vaccine approach of using a live, attenuated virus to activate both humoral and cellular immunity is not viable with HIV since the potential for reversion to a wild-type, pathogenic virus cannot be discounted (Baba et al., 1995; Letvin, 1998; McMichael, Mwau, and Hanke, 2002).
A recombinant gp120-based subunit vaccine has recently completed Phase III clinical trials in the USA and Thailand. This vaccine consists of a bivalent subtype B/B vaccine (USA trials) and a bivalent subtype B/E vaccine (Thailand) to target the predominant subtype in that geographical area (Berman et al., 1999; Billich, 2001; Francis et al., 1998). However, despite promising initial immunogenicity, broadly reactive neutralising antibody responses have been difficult to induce, and some individuals immunised in early phase I and II trials have subsequently succumbed to infection (Francis et al., 1998; Mwau and McMichael, 2003). As thought by many, this vaccine does not induce cross-reactive antibody and CTL responses, and trials with this vaccine have ceased (Francis et al., 2003).

Instead of envelope-based immunogens, many vaccine strategies now aim to prime the immune system with a DNA plasmid encoding a viral antigen(s) such as Gag, and follow with the administration of a live non-pathogenic vector [such as modified vaccinia virus Ankara (MVA)], expressing the same antigen(s). One DNA-MVA vaccine currently in Phase I clinical trials, aimed at the HIV epidemic in Kenya, combines consensus subtype A Gag (p24/p17) sequences with 25 known subtype A-specific CTL sequences (Hanke and McMichael, 2000; Hanke et al., 2002; Mwau and McMichael, 2003). Preliminary results from this study demonstrate a good stimulation of T-cells, with cross-clade activity observed in some instances, but no antibody production (McMichael, Mwau, and Hanke, 2002; Mwau and McMichael, 2003). Overall, there is much active research into the development of an HIV vaccine, but the continued lack of understanding of the immune correlates that provide prolonged protection against disease, as well as the issue of global HIV diversity means the future of controlling the HIV epidemic with a vaccine remains a huge challenge.

1.8.2 Highly active antiretroviral therapy (HAART)

There are currently 19 anti-HIV drugs and combination pills approved for use as HIV therapeutics, falling into three main categories (Table 1.5)(Cohen, 2002a). Nucleoside reverse transcription inhibitors (NRTIs e.g. zidovudine) are chain-terminating nucleoside analogues that become incorporated into the viral DNA and prevent replication of the viral genome. Non-nucleoside RT inhibitors (NNRTIs e.g. nevirapine) also inhibit reverse transcription, but by physically binding to a pocket on the enzyme (Fig. 1.7). The final class of inhibitor targets HIV PR (PIs e.g. indinavir)(Yeni et al., 2002). Since the introduction and widespread use of PIs in 1996,
combination antiretroviral therapy has dramatically increased the life expectancy of infected individuals. Early studies with indinavir, taken in combination with the two NRTIs zidovudine (AZT) and lamivudine, reduced plasma viral RNA loads to less than 50 copies per ml for up to 1 year in patients already treated with antiretrovirals (Gulick et al., 1997; Hammer et al., 1997). In addition to the enzyme inhibitors, a fusion inhibitor (enfuvirtide) is now approved for therapeutic use, but will be discussed in section 1.8.3.2.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Side Effects</th>
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<tr>
<td><strong>NRTI</strong></td>
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<tr>
<td>Abacavir</td>
<td>Lactic acidemia, induced by mitochondrial toxicity</td>
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<tr>
<td>Didanosine (ddl)</td>
<td>Hepatotoxicity</td>
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<tr>
<td>Lamivudine (3TC)</td>
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<td>Stavudine (D4T)</td>
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<td>Tenofovir</td>
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<td>Zalcitabine (ddC)</td>
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<td>Zidovudine (AZT)</td>
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<tr>
<td>Abacavir + Lamivudine</td>
<td>Hepatotoxicity</td>
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<tr>
<td>+ Zidovudine (Trizivir)</td>
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<td>Lamivudine + Zidovudine</td>
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<tr>
<td>(Combivir)</td>
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<td><strong>NNRTI</strong></td>
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<tr>
<td>Delavirdine</td>
<td>Hepatotoxicity</td>
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<tr>
<td>Efavirenz</td>
<td>Efavirenz causes teratogenicity (animals), mood swings,</td>
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<tr>
<td>Nevirapine</td>
<td>hallucinations and vivid dreams/nightmares.</td>
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<tr>
<td><strong>PI</strong></td>
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<tr>
<td>Amprenavir</td>
<td>Lipid abnormalities</td>
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<tr>
<td>Indinavir</td>
<td>Potential glucose intolerance</td>
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<tr>
<td>Lopinavir + Ritonavir</td>
<td>Hepatotoxicity</td>
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<tr>
<td>Nelfinavir</td>
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<tr>
<td>Ritonavir</td>
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<td>Saquinavir</td>
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<tr>
<td><strong>Fusion Inhibitor</strong></td>
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<tr>
<td>Enfuvirtide (T20)</td>
<td>Hepatotoxicity, nausea, fever, bacterial pneumonia</td>
</tr>
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Table 1.5. Current anti-HIV drugs and side effects. Current antiretroviral drugs approved for therapy. Side effects based upon those described in (Yeni et al., 2002).

Highly active antiretroviral therapy (HAART) is the term given to current combination therapy, which includes a number of regimens with a combination of PIs,
NNRTIs and/or NRTIs. Such regimens have proven to be highly effective at reducing viral loads below the level of detection and increasing the life span of infected individuals (Gulick et al., 1997; Hammer et al., 1997; Palella et al., 1998). In addition, a restoration of both CD4+ and CD8+ cell counts is achieved, resulting in at least a partially restored immune system (Kaufmann et al., 2000). The combination of drugs to prescribe as initial therapy, as well as the actual stage at which to begin antiretroviral therapy, remains a complex issue (Harrington and Carpenter, 2000; Yeni et al., 2002). Many factors need to be considered, including the stage of disease to start treatment, and the toxicity of the drugs (Yeni et al., 2002). Due to the rapid clearance of the majority of these inhibitors, high doses and multiple daily administrations are required. This complex regimen, in combination with often-severe side effects (Table 1.5) makes adherence to therapy extremely difficult, and is a major contributory factor to therapy failure (Tsasis, 2001; Volberding, 2002).

In addition to the severe side effects of antiretrovirals (Table 1.5) there are other drawbacks to HAART. A pressing concern is the frequent development of resistance. In particular, single amino acid mutations in RT can confer resistance to the whole class of NNRTIs, often leading to a therapy failure. Once therapy is stopped, patients are at risk of viral rebound, which may be caused by the activation of long-lived latently infected, resting CD4+ T-cells, and/or replication and release of virus from cells less sensitive to HAART, such as macrophages, virus-trapping FDCs, and cells within immunoprivileged sites (Blankson, Persaud, and Siliciano, 2002; Brack-Werner, 1999; Bukrinsky et al., 1991; Chun et al., 2000; Hoetelmans, 1998; Pomerantz, 2002; Ramratnam et al., 2000; Sharkey et al., 2000; Yeni et al., 2002). The length of time an individual has been on HAART, the history of previous regimen failures, and the finite number of therapeutic drug combinations make resistance a growing problem (Hammer et al., 1997; Trachtenberg and Sande, 2002; Yeni et al., 2002). As a final consideration, the improvements in general health achieved with HAART can re-activate sexual activity in HIV-infected individuals, leading to increased transmission of drug resistant virus.

There are many attempts underway to improve the therapy available for patients, one of which is the development of new drugs to help combat the ever-increasing intra-class resistance, with fewer and less severe side effects, better durability in the body to help reduce doses. One such newly approved drug is tenofavir disoproxil fumarate
(TDF), a nucleotide RT inhibitor (NtRTI) with a high potency and a long intracellular half-life, allowing a once-daily dosing (Squires, 2001). Changes in the therapeutic regimens are also under study. STIs in the early stages of disease increase HIV-specific CD8+ T-cell responses, and allow time off the toxic therapy. In addition, the virus population rapidly reverts back to a WT genotype upon cessation of therapy, increasing the chances of success of salvage therapies, if required upon resistance development (Hirschel, 2001; Miller, 2001). However, interruption of therapy in patients in later stages of disease and/or close to failing therapy is not as beneficial, as decreases in CD4+ cell counts and increases in viral load result. Even though there are some drawbacks to current HIV therapy as it stands, it remains an extremely valuable tool in the fight against AIDS.

1.8.3 Other approaches

HAART has dramatically improved the life of HIV-infected individuals, but there remains an urgent need for new drug targets (De Clercq, 2002). Current anti-HIV therapeutic strategies rely upon the inhibition of the viral RT and PR enzymes, and although these are very effective, there are several other steps in the viral life cycle at which future intervention strategies will be aimed (section 1.5 and Fig. 1.7). New inhibitors are currently in development, some of which are classical enzyme inhibitors and some of which target HIV entry (Gulick, 2003; Starr-Spires and Collman, 2002). Novel HIV entry therapeutics are discussed below.

1.8.3.1 Coreceptor inhibitors

HIV coreceptors, specifically CCR5 and CXCR4, are major targets for novel anti-HIV therapeutics (Starr-Spires and Collman, 2002). Synthetic small molecules that target HIV coreceptors are advantageous over natural chemokines as they do not induce inflammatory responses, and are more cost-effective for large-scale production. The first coreceptor-specific, small molecule inhibitor of HIV was the bicyclam compound AMD3100 (previously JMD3100 and SAD791) (De Clercq et al., 1992; De Clercq et al., 1994). The target of this molecule was known to block an early step within the viral life cycle, which was later identified as CXCR4-binding (De Clercq et al., 1994; De Vreese et al., 1996; Donzella et al., 1998; Feng et al., 1996; Hatse et al., 2002; Schols et al., 1997a; Schols et al., 1997b). AMD3100 is proposed to inhibit gp120:CXCR4 interactions by binding to residues in ECL2, TM4 and TM6 of X4, and blocking the electrostatic interaction of T-tropic envelopes as a result of the positive charge of the
two multicyclic polyamine cyclams (Fig. 1.13A) (De Clercq, 2000a; Labrosse et al., 1998).

**Figure 1.13** Small molecule coreceptor inhibitors and interaction with CCR5. Chemical structures of the HIV coreceptor small molecule inhibitors AMD3100 (A) and TAK-779 (B). Amino acid structure of CCR5 highlighting residues important for TAK-779 binding (red), and putative disulfide bridge positions (red lines) (C). Theoretical aerial view of CCR5 transmembrane domains, based upon the structure of rhodopsin, showing residues important for TAK-779 binding and the proposed binding pocket formed between TM 1, 2, 3, and 7 (D). Structure of a second CCR5 inhibitor, SCH-C shown in panel (E).
Two other CXCR4 inhibitors have been described. T22, a synthetic cationic 18-amino acid derived from peptides in the blood of the horseshoe crab, binds predominantly to negatively-charged residues in ECL1 and 2 of CXCR4 crucial for HIV envelope-binding (Murakami et al., 1997; Murakami et al., 1999). The differential sensitivities of X4 and R5X4 viral strains to this inhibitor is partially explained by the use of multiple regions of CXCR4 for HIV entry (Murakami et al., 1999; Owen et al., 2002) (McKnight et al., 1997; Strizki et al., 1997) (section 1.7.3). ALX40-4C [N-α-acetyl-nona-d-arginine (Arg) amide], is a stabilised cationic polypeptide containing 9 arginine residues also targeting CXCR4. ALX40-4C inhibits T- but not M-tropic isolates of HIV-1 on CXCR4 but not CCR5-expressing indicator cell lines (Sumner-Smith et al., 1995) (Doranz et al., 1997a). The precise mode of action remains undetermined, although this molecule does not induce receptor down-modulation (Doranz et al., 2001; Doranz et al., 1997a).

Of the CXCR4 inhibitors described above, both AMD3100 and ALX40-4C have undergone clinical trials. CXCR4 is the only receptor for the chemokine SDF-1α, and CXCR4 or SDF-1α knock-outs affect early development and are fatal in mice (Nagasawa et al., 1996; Tachibana et al., 1998; Zou et al., 1998). As such, the use of CXCR4 inhibitors in vivo deserves caution. Indeed, although no toxicity was observed with AMD3100 in early SCID-hu Thy/Liv mouse experiments, and phase I clinical trials showed a good absorption and tolerance of a single-dose administrated subcutaneously, clinical trials with AMD3100 have been halted due to poor oral bioavailability (Datema et al., 1996; Hendrix et al., 2000). A second-generation CXCR4 antagonist, AMD070, is in phase I clinical trials (Schols et al., 2003). Phase I/II clinical trials were carried out with ALX40-4C whilst it was believed to be an inhibitor of the Tat-TAR interaction (Sumner-Smith et al., 1995), yet important information regarding the safety of CXCR4-specific inhibitors was gained, demonstrating that molecules targeting this chemokine receptor may not be as toxic as first feared.

Several small molecule inhibitors of CCR5 have also been reported. TAK-779, an anilide derivative with quaternary ammonium moieties, is a potent CCR5 antagonist and inhibitor of R5-using HIV and SIV strains, yet demonstrates cross-reactivity with CCR2b (Fig. 1.13B) (Baba et al., 1999; Dragic et al., 2000; Shiraishi et al., 2000). TAK-779 binds to a small pocket formed between transmembrane helices 1, 2, 3 and 7

82
of CCR5, and the insertion of protruding hydrophobic residues on TAK-779 into the binding pocket of CCR5 may render it inflexible and prevent a successful interaction with gp120 (Fig. 1.13C and D; Chapter 3) (Dragic et al., 2000; Takashima et al., 2001). The second generation TAK-220 demonstrates a higher degree of coreceptor specificity than TAK-779, as well as more potent anti-HIV activity (Iizawa et al., 2003). Piperidine-based CCR5 inhibitors developed at Merck Research Laboratories also bind to a small pocket within CCR5, with particular interactions with transmembrane domains 2, 3, 6 and 7, but these are much less potent even that TAK-779 (Castonguay et al., 2003).

A second potent CCR5 inhibitor, SCH-C, has a similar chemical structure to TAK-779 (Fig. 1.13E); however, unlike TAK-779, this piperazine-based CCR5 antagonist does not bind to, or inhibit infection via, CCR2b (Strizki et al., 2001; Tagat et al., 2001). The mechanism of action of SCH-C has not been established, but the structural similarity to TAK-779 may indicate a similar mode of action. Early studies with a SCID-hu Thy/Liv mouse model suggested promising bioavailability and pharmacokinetics, yet subsequent phase I clinical studies demonstrated a lengthening of the hearts rhythmic cycle (QTc) at high doses (Este, 2002; Strizki et al., 2001). Second-generation compounds, such as SCH-350634, have now been produced and are undergoing preclinical toxicology tests (Este, 2002). A third recently described CCR5 inhibitor is UK-427857, a heterocycle-substituted azabicycloalkane isolated from a library of compounds able to inhibit MIP-1β binding to CCR5 via high throughput screening (Dorr et al., 2003). This inhibitor possesses extremely potent (IC50 <10 nM) and highly CCR5-specific cross-clade activity against a large range of both TCLA and primary HIV-1 isolates (Chapter 3) (Napier et al., 2003). Preliminary clinical trials indicate minimal toxicity and excellent bioavailability, making it a promising candidate for therapeutic use (Abel et al., 2003).

CCR5/CXCR4 inhibitors are thus promising therapeutic candidates. However, there are at least ten other 7TM receptors able to support infection by HIV/SIV in vitro (Table 1.4). The role of such alternative coreceptors during an in vivo infection is currently thought to be insignificant; but it will be critical to monitor coreceptor use during clinical trials (Lee et al., 2000; Zhang, Y. et al., 1998b). However, in vitro studies with AMD3100 found that viral escape to replicate in the presence of this drug evolved via mutations primarily in the V3 loop which changed gp120 conformation, but
was not associated with a coreceptor switch (De Vreese et al., 1996; De Vreese et al., 1997; Este et al., 1996). Resistance to the CCR5 inhibitor SCH-C took multiple viral passage over a 19-week period, yet was caused by virus variants that evolved, to utilise a different region of CCR5 (Trkola et al., 2002). It is promising that a coreceptor switch to CXCR4, and the associated increase in pathogenicity, has not been demonstrated to occur thus far.

1.8.3.2 Fusion inhibitors

Therapeutic methods that block the fusion of viral and cellular membranes are also under development. T20 (formerly DP178) is a 20-amino acid peptide derived from the helical C-terminal amphipathic heptad repeat in the ectodomain of gp41, which binds to the N-terminal leucine/isoleucine zipper coiled coil and prevents formation of the six-helix bundle and subsequent membrane fusion (section 1.7.4)(Furuta et al., 1998; Kliger and Shai, 2000; LaBranche et al., 2001; Salzwedel, West, and Hunter, 1999; Wild et al., 1994). As T20 is a peptide, it is expensive to produce, is rapidly cleared from the body, and induce a host antibody response (LaBranche et al., 2001). Anti-T20 mabs appear to have no detrimental effect, and early clinical trials were successful (Cohen et al., 2002; De Clercq, 2000b; Kilby et al., 2002; LaBranche et al., 2001). This peptide is very potent, blocking fusion with concentrations as low as 2 ng/ml, and is now available in limited supply as the drug enfuvirtide (Chen, Kilby, and Saag, 2002). Primary HIV strains are variable in their sensitivity to T20, with some reports suggesting an influence of coreceptor use; however, whether R5 or X4 strains are more sensitive remains unclear (Labrosse et al., 2003; Reeves et al., 2002).

Triterpenes also possess fusion inhibitory action. Some derivatives of betulinic acid carriedanti-PR activity; however, subsequent studies on modified compounds, such as RPR103611, had lost activity. Such compounds showed potent inhibition of HIV-1 (but not HIV-2) membrane fusion (Mayaux et al., 1994). Again, the precise mode of action of RPR103611 is not known; however, further investigations and/or clinical trials are justified.

1.9 Scope of Thesis

When HAART, consisting of RT and PR inhibitors, was introduced, it revolutionised HIV therapy, and has increased the life span of many infected individuals. However, the severe side-effects and complex drug regimens, in addition
to long-term use forcing the evolution and transmission of multi-resistant variants, often result in therapy failure. As a result, there is an urgent need for novel therapeutics targeting alternative stages of the HIV life cycle. One major area of interest is the development of HIV entry inhibitors, such as molecules blocking coreceptor binding or membrane fusion.

Chapter 3 of this thesis investigates a series of six novel heterocycle-substituted azabicycloalkane small molecule inhibitors of CCR5. Chemokines and chemokine receptors have crucial roles in the regulation of the immune system, and are often promiscuous in their use of chemokine receptors. As such, the specificity of chemokine receptor-targeting molecules is crucial, in order to reduce the risk of unwanted blockade of other chemokine receptors and potential immunosuppressive effects. Thus, the effect of these CCR5 inhibitors on indicator cell lines expressing a range of HIV/SIV coreceptors was evaluated. In addition, the capacity of these molecules to inhibit infection of a range of cell types (including PBMCs and macrophages) by a diverse selection of HIV and SIV strains was evaluated.

HIV and SIV predominantly utilise two coreceptors, CCR5 and CXCR4, for infection in vivo. Up to eighteen other chemokine receptors and related 7TM receptors have been shown to support HIV/SIV infection in vitro; however, their role during infection is currently thought to be insignificant. However, the use of coreceptor inhibitors as HIV therapeutics may force the evolution of HIV variants able to efficiently use these alternative coreceptors. Chapter 4 of this thesis therefore investigates the expression of functional alternative coreceptors on a range of primary, untransformed cell types. The ability of a panel of HIV and SIV strains to infect brain microvascular endothelial cells, astrocytes, Leydig cells and PBMCs lacking CCR5 was assessed. The nature of the alternative coreceptor was investigated by inhibition with a range of chemokines, antibodies and small molecule inhibitors.

Approximately 50% of subtype B isolates undergo an expansion in coreceptor use during disease progression, where they switch from being R5 to R5X4 or X4. In these cases, this switch is associated with the onset of AIDS. However, subtype C-infected individuals undergo a rapid progression to AIDS despite the rare evolution of CXCR4-using strains. It is thus possible that in these instances HIV evolves to utilise alternative coreceptors, but not CXCR4. Such a scenario could have implications in the
advent of CCR5 inhibitors. Chapter 5 investigates the relative frequency of CXCR4-use in a large panel of subtype B and C isolates. In addition, the capacity of such isolates to infect primary cells, demonstrated in Chapter 4 to express a functional alternative coreceptor, was evaluated. The capacity of subtype B and C strains to use alternative coreceptors on indicator cell lines, and infect primary cells in the absence of CCR5, was correlated with sequences of the third variable loop (V3) of the HIV envelope.
CHAPTER 2

Materials and Methods

2.1 Buffers and Solutions

All buffers and solutions used are described in Table 2.1.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA coating buffer</td>
<td>0.1 M NaHCO₃; 0.15 M NaCl, pH 8.5</td>
</tr>
<tr>
<td>Formol saline</td>
<td>0.5% NaCl; 1.5% Na₂SO₄; 40% formaldehyde</td>
</tr>
<tr>
<td>LB-broth (Luria-Bertani)</td>
<td>1% Bacto-tryptone; 0.5% Yeast extract; 1% NaCl</td>
</tr>
<tr>
<td>LB-agar</td>
<td>1% Bacto-tryptone; 0.5% Yeast extract; 1% NaCl;</td>
</tr>
<tr>
<td></td>
<td>1.5% Agar</td>
</tr>
<tr>
<td>Orange G loading buffer</td>
<td>50% glycerol; Orange G (Sigma)</td>
</tr>
<tr>
<td>Phosphate-buffered saline (PBS)</td>
<td>0.8% NaCl; 0.025% KCl; 0.143% Na₂HPO₄; 0.25% Kh₂PO₄</td>
</tr>
<tr>
<td>20x sodium citrate (SSC)</td>
<td>3 M NaCl; 300 mM sodium citrate</td>
</tr>
<tr>
<td>Tris-acetate EDTA (TAE)</td>
<td>20 mM Tris-HCl pH 7.8; 20 mM sodium acetate; 1 mM EDTA</td>
</tr>
<tr>
<td>Tris-buffered saline (TBS)</td>
<td>20 mM Tris-HCl pH 7.6; 120 mM NaCl</td>
</tr>
<tr>
<td>Versene</td>
<td>0.8% NaCl; 0.02% KCl; 0.11% Na₂HPO₄; 0.02% Kh₂PO₄; 0.02% versene (EDTA); 0.15% phenol red</td>
</tr>
</tbody>
</table>

Table 2.1. Composition of buffers. Composition of all buffers used within this study are described.

2.2 Nucleic Acid Production and Manipulation

2.2.1 Restriction enzyme digestion of DNA

Restriction enzymes were obtained from Promega Life Sciences, and used in the recommended buffers according to manufacturers' instructions. Enzyme concentrations were 10 units of enzyme per µg of DNA.

2.2.2 Agarose gel electrophoresis of DNA

Agarose gel electrophoresis was performed to separate DNA fragments by size, and to purify oligonucleotide products of polymerase chain reaction (PCR; section 2.2.10). Agarose gels were made with 0.8-2% w/v agarose (Anachem-Scotlab) in 0.5 x TAE buffer, according to fragment size. The agarose was dissolved before adding ethidium bromide (0.5 µg/ml) and pouring into an appropriate gel tray with 'well-
forming' comb in place. Once solidified, the gel was transferred to a flat bed gel electrophoresis chamber (Labnet International, Inc.) and submerged in 0.5 x TAE buffer. Samples were mixed with 0.3 volumes loading buffer and loaded into the gel alongside a molecular weight marker of the appropriate size (100 bp or 1 kb; MBI Fermentas). DNA was electrophoresed at a voltage and time length dependent upon the gel density and size of fragments to be separated, and visualised by illumination with short wave ultraviolet (UV) light.

2.2.3 Isolation of DNA from agarose gels

Following fractionation, specific DNA bands were visualised by long wave UV light and excised from the gel with a clean scalpel before purifying with the QIAquick Gel Extraction Kit (QIAGEN) according to manufacturers’ instructions. In brief, the gel slice was melted at 50°C in 3 gel volumes of buffer QG, a high-salt and pH-indicating solubilisation and binding buffer. DNA was bound to the silica membrane in the QIAquick column by applying a vacuum. The column was then washed with 500 μl buffer QG and 750 μl of ethanol-containing buffer PE to remove traces of agarose gel and excess salts respectively. DNA was then eluted in low-salt buffer PB or milliQ water. Yield and purity of DNA was confirmed by agarose gel electrophoresis.

2.2.4 Ligation of DNA fragments

2.2.4.1 Overnight ligation

Ligations were performed in a total volume of 10 μl, with 1 μl T4 DNA ligase, 1 μl of 10x T4 DNA ligase buffer (both Promega) and molar ratios of vector to insert DNA of 1 to 3. Control ligations, where insert DNA was replaced by water, were carried out in parallel to estimate the background levels of vector religation. Success of ligation was determined by transformation into the competent bacteria Top 10F (Invitrogen) and analysis as described in sections 2.2.6 and 2.2.7.

2.2.4.2 Rapid ligation

A rapid ligation of insert into vector was done using the Rapid DNA Ligation Kit (Roche Diagnostics). The ligation mix contained vector and insert DNA at a molar ratio of 1:3 - 1:5 in a total volume of 10 μl DNA dilution buffer, 10 μl 2x T4 DNA ligation buffer and 1 μl T4 DNA ligase. After 10 minutes at room temperature the
reaction was transformed into competent bacteria (section 2.2.7). Control ligations to estimate the background levels of vector re-ligation were carried out as described above.

2.2.4.3 Ligation of purified PCR products into cloning vectors

Gel-purified PCR products were ligated directly into the linearised cloning vector pGEM®-T Easy (Promega). This vector possesses overhanging T residues to enable an easy ligation with PCR products, which have complementary overhanging A's. Reactions were set up in a total volume of 10 µl, containing 5 µl of 2x T4 DNA ligation buffer, 1 µl of T4 DNA ligase (3 units), 1 µl of vector DNA (50 ng) and a volume of purified PCR product resulting in a 1:3 vector:insert molar ratio. Positive and negative control ligations were carried out in parallel, with 2 µl of control insert DNA or vector alone for the positive and negative control reactions, respectively. Ligations were incubated for 1 hour at room temperature before transforming competent bacteria.

2.2.5 Preparation of competent bacteria

A culture of an existing competent bacterial cell stock (Top 10 F', Invitrogen) was grown in 5 ml of LB broth overnight and used to inoculate 500 ml LB broth. This culture was grown at 37°C in an orbital shaker rotating at 250 min⁻¹ until optical density at 600 nm (OD₆₀₀) reached 0.5. Growth was rapidly halted by shaking the flask in iced water, and cells were pelleted by centrifuging at 4°C for 15 minutes at 4200 rpm. Bacterial pellets were resuspended in 30 ml sterile, ice-cold 100 mM CaCl₂, incubated on ice for 30 minutes, and pelleted at 4000 g/4°C. Cells were gently resuspended in 5 ml ice-cold sterile 100 mM CaCl₂ with 15% glycerol (weight/volume), aliquoted in sterile 0.5 ml microcentrifuge tubes in a dry ice:ethanol bath and stored at -80°C until use. Competency was determined by transforming with 1 ng undigested plasmid vector and calculating the number of colonies per µg of DNA, typically between 5 x 10⁶ and 2 x 10⁷.

2.2.6 Transformation of competent bacteria

Bacterial cells were transformed by adding 1-2 ng plasmid DNA (or 100 ng ligation mix) to a 15 ml polypropylene tube on ice. Competent cells were thawed on ice before adding 50 µl to 1 µl plasmid DNA and incubating on ice for 30 minutes. Cells were then heat-shocked at 42°C for 45 seconds, incubated for a further 2 minutes on ice.
and recovered by shaking at 200 rpm for 1 hour in 500 μl LB. Cultures were then plated on L-agar plates containing 100 μg/ml ampicillin and other appropriate selection markers (section 2.2.7.1).

2.2.7 Screening of transformants

2.2.7.1 Colour selection

Cloning vectors like pGEM® T-Easy contain the β-galactosidase (β-gal) gene and allow screening by colour. Successful vector:insert ligation interrupts the β-gal gene and produces white colonies when plated on L-agar plates containing 100 μg/ml ampicillin, 0.5 mM IPTG and 80 μg/ml X-gal. Unsuccessful ligations produce blue colonies. Positive colonies were subjected to further analysis by restriction digestion and/or PCR.

2.2.7.2 PCR

Bacterial colonies were screened by PCR for the presence of the insert. An individual colony was picked and used to inoculate a 50 μl PCR reaction mixture containing primers annealing to (i) sites flanking the vector cloning site, to amplify the entire insert, (ii) sites within the insert itself or (iii) one site within the insert and one flanking the vector cloning site, to determine orientation. Positive colonies were then subjected to restriction digestion analysis and/or sequencing, following extraction of plasmid DNA.

2.2.7.3 Restriction enzyme digestion

Plasmid DNA isolated from individual colonies was analysed by restriction digestion with the appropriate enzymes in order to determine both presence and orientation of the insert.

2.2.8 Small-scale isolation of plasmid DNA

A single bacterial colony from an agar plate or 2 μl of an existing bacterial glycerol stock was used to inoculate 4 ml of LB broth containing ampicillin, which was grown overnight in an orbital shaker at 37°C, rotating at 220 min⁻¹. The next day a glycerol stock was made (if not already existing) by adding 500 μl of the culture to 500 μl sterile 40% glycerol. The remaining 3.5 ml was centrifuged at 4000 g for 10 minutes
at 4°C, before removing supernatant and preparing plasmid DNA using the QIAprep Spin Miniprep Kit (QIAGEN). The pellet was resuspended in 250 μl RNaseA-containing buffer P1 and cells lysed under alkaline conditions by adding 250 μl buffer P2 (NaOH/SDS) and gently inverting the tube 5-6 times. The alkaline lysate was neutralised and adjusted to high salt conditions by the addition of 350 μl buffer N3 and inverting gently 5-6 times. The SDS-genomic DNA precipitate was pelleted by centrifugation at 13000 g for 10 minutes. Supernatant was collected and plasmid DNA isolated by passage through the QIAGEN columns, containing an anion exchange resin, on a vacuum pump. Columns were washed as according to manufacturer's instructions and centrifuged at 13000 g for 1 minute to remove residual wash buffer. DNA was eluted in 50 μl sterile MilliQ water and the concentration of nucleic acid was determined by measuring absorbance of the solution at 260 nm in a UV spectrophotometer. An absorbance of 1 cm⁻¹ is equivalent to 50 μg/ml for DNA.

2.2.9 Automated DNA sequencing

Sample DNA was sequenced by adding 250 ng of plasmid preparation to 2 μl of ddH₂O, 1 μl of an appropriate primer at 3-5 pmol and 4 μl of the sequencing premix (Beckman) in a sterile thin-walled 0.5 ml tube. Reactions were run on an MJ Research PTC 200 thermal cycler with a heated lid. Resulting DNA products were cleaned by transferring to a sterile eppendorf tube and adding 2 μl 3M NaOAc, 2 μl 100 mM EDTA, 1μl glycogen and 10 μl ddH₂O. Tubes were mixed well before 60 μl of ice-cold 95% EtOH was added. After vortexing, samples were left on ice for 5-10 minutes before centrifuging in a microfuge for 15 minutes at 13000 g and 4°C. Supernatant was carefully removed, and the pellet washed in 200 μl of cold 70% ethanol. After all ethanol was removed, pellets were left to air-dry at room temperature and resuspended in 40 μl of deionised formamide before loading into a sequencing sample plate, overlaying with a drop of mineral oil and running in a Beckman CEQ-2000 DNA Analysis machine. Sequences were analysed using the Sequencher software (Gene Codes Corporation, MI, USA).

2.2.10 Polymerase chain reaction (PCR)

PCR is a technique by which specific DNA sequences are amplified, used to screen transformed bacteria or to alter a DNA sequence i.e. add a restriction site. Reactions were carried out in 0.5 ml eppendorf tubes with 5 μl 10x Taq polymerase
buffer, 1 μl 40 mM dNTPs (10 mM each dATP, dTTP, dCTP, dGTP; Pharmacia
Biotech), 100 ng of the appropriate oligonucleotide primer, 0.5 μl of Taq polymerase (5
units/μl; Perkin-Elmer), 50-100 ng sample DNA or equal volume of sterile water, made
up to 50 μl in sterile MilliQ water. Reactions were overlaid with 20 μl irradiated
mineral oil (Sigma) and transferred to a thermal cycler (MJ Research PTC 200).
Following an initial denaturing step at 95°C for 3 minutes, the sample DNA was
subjected to thirty 3-step cycles consisting of a thirty second denaturation step at 95°C,
a thirty second primer-annealing step at 5°C below the given melting temperature (Tm)
and a DNA extension step for forty five seconds at 72°C. PCR products were cooled at
4°C before analysing by agarose gel electrophoresis (section 2.2.2).

2.2.11 RT-PCR

2.2.11.1 Isolation of total cellular RNA

Adherent cells were dislodged from tissue culture flasks with trypsin:versene,
counted and pelleted by centrifugation for 5 minutes at 210 g. Suspension cells were
counted and pelleted. Media was removed by aspiration, and total cellular RNA
extracted using the RNEasy kit from QIAGEN according to manufacturers’ directions.
Briefly, 3 x 10^6 – 1 x 10^7 cells were resuspended and disrupted in a high-salt denaturing
lysis buffer containing guanidine isothiocyanate (an RNase inhibitor), and homogenised
on a QIAshredder column (QIAGEN) by centrifuging at 13000 g for two minutes.
Sample was added to the RNA-binding column following the addition of 350 μl 70%
ethanol, to create correct resin-binding conditions, and centrifuged at 8000 g for 15
seconds. Flow-through was discarded, and bound RNA washed once in buffer RW1, and
twice in buffer RPE by centrifuging for 15 seconds at 8000 g. Following a final spin at
full speed for 1 minute to remove residual wash buffer, RNA was eluted in 30 - 50 μl
RNase-free water.

2.2.11.2 Purification of mRNA

mRNA was isolated from total cellular RNA using the Oligotex kit from
QIAGEN, which allows the isolation of poly-adenylated RNA on a dT resin coupled to
a solid-phase matrix. RNA was incubated with a high-salt buffer and the Oligotex bead
suspension at 70°C to disrupt secondary structure of RNA. mRNA was bound to the
beads by incubating at room temperature for 10 minutes before the bead suspension was
pelleted by centrifugation at 18000 g for 2 minutes, supernatant removed and the pellet
resuspended in 400 μl buffer OW2. After addition to a spin column and centrifuging for 1 minute at maximum speed, bound mRNA was washed once in buffer OW2 and eluted by resuspending the resin on the column in 20-100 μl hot, low ionic-strength buffer OEB and centrifuging for 1 minute at maximum speed.

2.2.11.3 RT-PCR amplification of specific mRNA

Specific mRNA sequences were amplified from mRNA by reverse transcriptase PCR (RT-PCR), a technique using reverse transcriptase to synthesise a cDNA strand, and a DNA polymerase to amplify the specific sequence by normal PCR.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5‘-3’</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>TGGATATTGCCATCAATGACC</td>
<td>457</td>
</tr>
<tr>
<td></td>
<td>GATGGCATGGACTGTGTCATG</td>
<td></td>
</tr>
<tr>
<td>CD4</td>
<td>TCAGGGAAAGAAAGTGTTGC</td>
<td>170</td>
</tr>
<tr>
<td></td>
<td>AAGAAGGCCCTGATTCCC</td>
<td></td>
</tr>
<tr>
<td>CCR5</td>
<td>CTTCAATTACACTGCAGCTCT</td>
<td>182 (Wild-type)</td>
</tr>
<tr>
<td></td>
<td>CACAGCCTGTGCTGCTTCTTCTT</td>
<td>150 (Δ32)</td>
</tr>
<tr>
<td>CXCR4</td>
<td>TAGATATCTTTACCAGGGGGGATCAG</td>
<td>1044</td>
</tr>
<tr>
<td></td>
<td>TAGCAGCCGGTTAGCTGAGTGAAAACCTTG</td>
<td></td>
</tr>
<tr>
<td>CCR8</td>
<td>GCAAGTTGGCTCCTTGCTGTC</td>
<td>710</td>
</tr>
<tr>
<td></td>
<td>CATGGTGGGATAAAGTCAGC</td>
<td></td>
</tr>
<tr>
<td>CCR3</td>
<td>GCTGATACCCAGACACTGATG</td>
<td>834</td>
</tr>
<tr>
<td></td>
<td>CAACAAAGCCGCTGATCACCCG</td>
<td></td>
</tr>
<tr>
<td>GPR1</td>
<td>CCAGCTGGGAATTTGTGTTCTACT</td>
<td>1068</td>
</tr>
<tr>
<td></td>
<td>GCTGTTTCCAGTAGACACAG</td>
<td></td>
</tr>
<tr>
<td>GPR15</td>
<td>ATGGACCCAGAAAGAAACTTC</td>
<td>1100</td>
</tr>
<tr>
<td></td>
<td>TTAAGTGACACAGACCTC</td>
<td></td>
</tr>
<tr>
<td>CXCR6</td>
<td>CAGGCATCCATGAATGGGTTG</td>
<td>766</td>
</tr>
<tr>
<td></td>
<td>CAAGGCTATAAACTGGAACATGCTG</td>
<td></td>
</tr>
<tr>
<td>RDC1</td>
<td>AAGAAGATGTTACGCGGTGCTGCTGACTTCTG</td>
<td>280</td>
</tr>
<tr>
<td></td>
<td>CTGCTGCTGCTGCTCTGCTGCTGACCGTTC</td>
<td></td>
</tr>
<tr>
<td>HCR</td>
<td>TGAAGATGGCCAATTACACCGTG</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>CAGGATAAGCACAACCAGAGATTCG</td>
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</tr>
<tr>
<td>CX3CR1</td>
<td>ATGGATACGTTCCCTGAAATCACGAGAAAAC</td>
<td>1004</td>
</tr>
<tr>
<td></td>
<td>TCAGAGAAGGAGCAATGCTTCCATCACTCG</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.2. RT-PCR primer sequences. Sequences of primers used for the amplification of specific HIV and SIV receptors and coreceptors from whole cellular mRNA, and the sizes of DNA products are described.
The TITANIUM™ One-Step RT-PCR Kit (Clontech), enabling all reaction steps to occur in a single tube, was used according to manufacturers’ instructions. Briefly, a PCR master mix, containing 5 µl 10x One-Step buffer, 1 µl 50x dNTP mix, 0.5 µl recombinant RNase inhibitor (40 units/ml), 25 µl thermostabilizing reagent (reduces RNA folding), 10 µl GC-melt (destabilises G-C pairing), 1 µl Oligo (dT) primer and 1 µl 50x RT-TITANIUM Taq enzyme mix, was prepared. 43.5 µl of this master mix was added to 1 µl mRNA sample with 1 µl each primer at 45 µM, and brought up to a final volume of 50 µl with RNase-free water. Primer sequences for all mRNAs amplified are shown in Table 2.2. Reactions were cycled at 50°C for 1 hour and 94°C for 5 minutes, followed by 25 three-step cycles of 94°C for 30s, 65°C for 30s and 68°C for 1 minute, with a final elongation step at 68°C for 2 minutes. The positive control reaction was the amplification of the house-keeping gene glyceraldehyde 3'-phosphate dehydrogenase (GAPDH), and negative controls were primers for a gene known not be expressed in that cell type. All reactions were carried out in duplicate, with one sample added following the hour at 50°C during which reverse transcription occurs to demonstrate the absence of contaminating genomic DNA sequences.

2.2.12 Viral envelope V3 loop sequencing

Proviral DNA was amplified from infected PBMCs in order to sequence the envelope V3 loop. Cells were infected as described in section 2.4.2. At the peak of viral replication (day 7-10), cells were centrifuged at 250 g for 5 minutes, and supernatants discarded. DNA was removed from the cell pellet using the DNeasy Tissue Kit from QIAGEN. In brief, cells were lysed by resuspending in proteinase K-containing buffer and incubating at 70°C for 10 minutes. Buffer conditions were optimised for DNA-binding by the addition of 200 µl 70% ethanol, and DNA was applied to the silica gel membrane column. Following centrifugation at 13000 g for 1 minute, contaminating enzyme inhibitors and cations were removed by washing once with 500 µl each buffer AW1 and AW2. After drying the membrane by centrifugation, bound DNA was eluted twice in 200 µl of buffer AE.

V3 sequences were amplified from isolated cellular DNA by PCR, using primers as described in Table 2.3. Cycling parameters used were: 94°C 3 minutes, followed by 30 cycles of 94°C for 1 minute, 52°C for 1 minute and 72°C for 3 minutes, with a final
elongation step of 72°C for 10 minutes. Amplified sequences were purified by agarose gel electrophoresis, and cloned into the intermediate cloning vector pGEM®T-Easy (section 2.2.4.3). Primers annealing to the T7 and Sp6 sites within this vector were used for the sequencing reaction (section 2.2.9).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BF</td>
<td>TCAACATGGAATTAGCCAGTAGT</td>
</tr>
<tr>
<td>BR</td>
<td>TTCCCCCTCCACAATTAACACTGTG</td>
</tr>
<tr>
<td>CF</td>
<td>AACACAAGCCCTGCTCCAAAGGTAC</td>
</tr>
<tr>
<td>CR</td>
<td>TTCTCCTCTACAATTAAAGCTATG</td>
</tr>
<tr>
<td>V1V2A3</td>
<td>CCCATACATTATTGTGCC</td>
</tr>
<tr>
<td>V4R</td>
<td>GACCCAGAATTGTAATGC</td>
</tr>
</tbody>
</table>

Table 2.3. V3 loop amplification primers. Primer sequences used for the amplification of subtype B and C envelope V3 loop sequences.

2.3 Eukaryotic Cell Culture

2.3.1 Cell lines

Cell lines used and the specific selection media required are listed in Table 2.4. All adherent cell lines were grown in Dulbecco's Modified Eagles Medium (DMEM) containing 0.11 g/L Na Pyr with 10% heat-inactivated foetal calf serum (FCS, Helena Biosciences) and Penicillin-Streptomycin (Pen-Strep, Gibco Invitrogen Corporation, final concentration of 100 units/ml penicillin and 100 μg/ml streptomycin) unless stated otherwise. All suspension cells were maintained in Roswell Park Memorial Institute 1640 (RPMI 1640), containing L-glutamine and pen-strep. G418 is from Gibco Invitrogen Corporation and puromycin from Sigma Biochemicals.

2.3.2 Cell maintenance

All cells were grown in a humidified atmosphere at 37°C with 5% CO₂. Cells were maintained by passaging every 3 - 7 days at dilutions of 1:5 - 1:20 as required. For cells grown in T25 tissue culture flasks (Helena BioSciences), media was removed by aspiration and cells rinsed in 1 ml of versene/trypsin (0.05% trypsin in 0.02% versene) to remove FCS before adding 1 ml of trypsin/versene and incubating at 37°C to detach cells. Trypsin/versene was inactivated by the addition of 5 ml media containing FCS and cells were diluted as necessary.
<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Cell Type</th>
<th>Growth Medium</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>U87/CD4</td>
<td>Human glioma</td>
<td>DMEM, G418 100 µg/ml</td>
<td>(Bjorndal et al., 1997; Clapham, Blanc, and Weiss, 1991; Willett et al., 1997)</td>
</tr>
<tr>
<td>U87/CD4/CCR5</td>
<td></td>
<td>DMEM, G418 100 µg/ml Puromycin 1 µg/ml</td>
<td>(Deng et al., 1997)</td>
</tr>
<tr>
<td>U87/CD4/CCR2b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NP2/CCR5</td>
<td>Human glioma</td>
<td>DMEM, G418 100 µg/ml Puromycin 1 µg/ml</td>
<td>(Soda et al., 1999)</td>
</tr>
<tr>
<td>NP2/CD4/CCR5</td>
<td></td>
<td>DMEM, G418 100 µg/ml Puromycin 1 µg/ml</td>
<td></td>
</tr>
<tr>
<td>NP2/CD4</td>
<td></td>
<td>DMEM, G418 100 µg/ml Puromycin 1 µg/ml</td>
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</tr>
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<td>DMEM, G418 100 µg/ml Puromycin 1 µg/ml</td>
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</tr>
<tr>
<td>GHOST-GPR15</td>
<td>Human osteosarcoma</td>
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</tr>
<tr>
<td>GHOST-CXCR6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
<td>DMEM</td>
<td></td>
</tr>
<tr>
<td>MOLT4 (clone 8)</td>
<td>T cell, T4 lymphoblastoid</td>
<td>RPMI</td>
<td>(Daniel et al., 1988; Kikukawa et al., 1986)</td>
</tr>
<tr>
<td>293T</td>
<td>Human fibroblast</td>
<td>DMEM</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.4. Cell lines. Cell lines used in this study, with details of selective growth media, and original references.

2.3.3 Freezing cells

Cells were fed with fresh medium the day before freezing, dislodged with trypsin and resuspended in 10 ml of medium before counting. The cell suspension was centrifuged at 210 g for 10 minutes and the cell pellet resuspended in cold medium with 20% FCS at a concentration of $1 \times 10^7$/ml before adding an equal volume of cold DMEM/20% FCS/20% dimethyl sulphoxide (DMSO) dropwise. Cells were aliquoted in cryovials and slowly frozen overnight at -80°C before transferring to vapour phase liquid nitrogen for long-term storage.
2.3.4 Thawing cells

Frozen cell aliquots were incubated at 37°C until just thawed before diluting in 10 ml of cold medium with 10% FCS. Cells were pelleted at 210 g for 10 minutes, washed in 10 ml of fresh growth medium and centrifuged again at 210 g for 5 minutes. The cells were grown in 10 ml of selection-free media at 37°C until stabilised.

2.3.5 Preparation of primary cells

2.3.5.1 Preparation of peripheral blood mononuclear cells (PBMCs)

Fresh blood (buffy coats from Hospital Transfusion Services, Brentwood) was diluted 1:1 in RPMI 1640 with L-glutamine (Gibco Invitrogen Corporation UK) and 30 ml was layered on 15 ml sterile ficoll (Lymphoprep, Nycomed, Oslo, Norway) in a 50 ml Falcon tube before centrifuging for 30 minutes at 700 g. Erythrocytes pellet at the bottom of the tube and white cells form a band at the interface between the ficoll and the plasma/PBS. The white cell layer was carefully removed with a pipette and washed twice by diluting 1:1 in RPMI 1640 and spinning at 250 g for 10 minutes. The final pellet was resuspended in RPMI 1640, 20% FCS and phytohaemagglutinin (PHA, 1 µg/ml; Sigma Chemicals) at 2 x 10^6 cells/ml and grown for 2-3 days to activate the lymphocytes. Cells were then diluted to 1 x 10^6/ml and grown in media containing 20% FCS and interleukin-2 (IL-2, Roche; 20 units/ml) for 2-3 days before use. PBMC from an individual homozygous for a 32 base-pair deletion in CCR5 were prepared from whole blood, drawn under ethical approval from the Committee on the Protection of Human Subjects in Research at the University of Massachusetts Medical School.

2.3.5.2 Preparation of macrophages

PBMCs were prepared from fresh blood as described above and resuspended at 1 x 10^7/ml in DMEM with 5% heat-inactivated human serum (Insight Biotechnology). Cells were plated in 10 - 12 ml volumes in 140 mm bacterial petri dishes and incubated for two hours at 37°C before washing gently in media three times and incubating overnight in DMEM/10% human serum. The next day cells were washed as above and incubated for 5-7 days before use.

2.3.5.3 Preparation of primary astrocytes

Foetal brain tissue was obtained from the Medical Research Council Tissue Bank (Hammersmith Hospital, London, UK). Adult astrocytes were gratefully received
from Jeanne Bell at the University of Edinburgh. All material was treated in compliance with institutional and ethical regulations approved by the Royal Marsden NHS Trust Research Ethics Committee. Tissue was washed once in cold HANKS buffered saline solution (BSS) and once in brain medium [DMEM/10% FCS/1% pen/strep/2 mM L-glutamine (Gibco Invitrogen Corporation)/1000 U/ml GM-CSF]. Tissue was then placed in a sterile culture dish to remove blood vessels with a scalpel before finely dissecting the tissue. Fragments were resuspended in brain culture medium and filtered through a 100 μm cell strainer (Falcon). Remaining cell clumps were repeatedly resuspended and strained until cleared. The cell suspension was pelleted in a centrifuge at 325 g for 10 minutes at 4°C, resuspended in brain culture medium at 0.5 - 1 x 10⁷ cells/ml and incubated for approximately 14 days to adhere, replacing GM-CSF and passaging when necessary. Astrocytes were selected from mixed brain cultures by washing in serum-free brain medium and agitating in a plate shaker (Dynax) at 37°C for 1-2 hours in order to detach microglia. Cells were washed once again in serum-free brain medium before adding astrocyte culture medium (as for brain medium but without GM-CSF) and incubating for 2 days at 37°C. Cultures were then passaged 1:2, incubated for a further two days and reshaken as described above. Following a further incubation for 2 days the purity of the astrocyte culture was determined by immunostaining for the astrocytic marker glial fibrillary acidic protein (GFAP; section 2.3.7.1).

2.3.5.4 Brain microvascular endothelial cells

Primary brain microvascular endothelial cells of a low passage number were purchased from Clonetics. Upon thawing, cells were maintained in Endothelial cell Basal Medium 2 (EBM-2) supplemented with 2% FCS, hydrocortisone, hFGF, VEGF, IGF, ascorbic acid, hEGF, GA-1000 antibiotic mix and heparin.

2.3.5.5 Preparation of primary Leydig cells

Leydig cells were prepared from testicular tissue obtained from elderly men suffering from hormono-dependent prostatic carcinoma, in compliance with local ethics committees, at GERM-INSERM U. 435 in Rennes, France, by Dr. Nathalie Dejucq as previously described (Willey et al., 2003b). Cells were maintained in 1:1 F-12/DMEM (15 mM NaHCO₃, 15 mM Heps-buffered Ham’s F-12 medium and DMEM).
2.3.6 Flow cytometry

2.3.6.1 Flow cytometry

Cells were detached in versene at 37°C, counted and placed in 5 ml polystyrene round-bottom tubes (Falcon) with $2 \times 10^5$ cells per sample. Samples were centrifuged at 250 g for 5 minutes and the cell pellet resuspended in 25 μl of 5% heat aggregated γ-globulin (HAG) in PBS/1% FCS/0.1% azide in order to prevent non-specific binding of antibodies. Cells were left for 20 minutes before adding 25 μl of primary antibody at twice the required concentration in PBS/1% FCS/0.1% azide. All antibodies used in this study are described in Table 2.5, and were used at 5 μg/ml unless stated otherwise. After incubating for 1 hour at room temperature, cells were washed twice in 2 ml PBS/1% FCS/0.1% azide by centrifuging for 5 minutes at 1200 rpm, and resuspended in 50 μl of the appropriate secondary antibody. For flow cytometry, all samples in this study used a goat anti-mouse FITC-conjugated antibody (Dako). Cells were incubated for a further hour before washing twice in 2 ml of PBS/1% FCS/0.1% azide and once with 2 ml of PBS. Cells were fixed as a single cell suspension in 500 μl of 2% formol saline. Analysis was carried out on a FACScan using the CellQuest software (BD Pharmingen).

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody Description</th>
<th>Supplier</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4</td>
<td>Clone Q4120; mouse anti-human</td>
<td>NIH AIDS Research and Reference Reagent Program</td>
<td>(Healey et al., 1990)</td>
</tr>
<tr>
<td>CCR3</td>
<td>Clone 7B11</td>
<td>NIH AIDS Research and Reference Reagent Program</td>
<td>(Heath et al., 1997)</td>
</tr>
<tr>
<td>CCR5</td>
<td>Clone 2D7; mouse anti-human</td>
<td>NIH AIDS Research and Reference Reagent Program</td>
<td>(Wu et al., 1997a)</td>
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<td>CCR5</td>
<td>Mab 182; PE-conjugated</td>
<td>R and D Systems</td>
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</tr>
<tr>
<td>CXCR4</td>
<td>12G5; mouse anti-human</td>
<td>NIH AIDS Research and Reference Reagent Program</td>
<td>(Endres et al., 1996)</td>
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<tr>
<td>GFAP</td>
<td>Rabbit monoclonal</td>
<td>Dako USA</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.5. Antibodies for flow cytometric analysis and immunostaining. Antibodies used for flow cytometry and immunostaining, as well as suppliers and appropriate references are described.

2.3.6.2 Quantitative FACS

The cell surface expression level of CCR5 was quantified by the use of the QuantiBRITE™PE phycoerythrin (PE) fluorescence quantitation kit (BD Pharmingen).
This system uses a pre-calibrated mix of beads conjugated with varying quantities of PE molecules to calibrate the FL2 fluorescence of a flow cytometer with respect to numbers of PE molecules. Staining cells with a CCR5-PE mab, with one PE molecule bound per antibody, the number of antibodies bound per cell (ABC) can be determined.

**A**

**B**

<table>
<thead>
<tr>
<th>Marker</th>
<th># PE bound per cell</th>
<th>Geo Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>1400</td>
<td>10.70</td>
</tr>
<tr>
<td>Med - Low</td>
<td>14000</td>
<td>119.58</td>
</tr>
<tr>
<td>Med - High</td>
<td>36600</td>
<td>332.99</td>
</tr>
<tr>
<td>High</td>
<td>182000</td>
<td>1739.38</td>
</tr>
</tbody>
</table>

**Figure 2.1. Quantitative FACS analysis.** Quantitation of cell surface-expressed proteins, indicating the forward and side-scatter properties of the control PE-conjugated beads (A), the relative fluorescence (FL2-H) of the four bead populations with differing levels of conjugated PE (B), and the correlation between number of PE molecule and geometric mean of fluorescence (C).

Cells were detached, counted and resuspended in 25 µl HAG as described above. After incubation at room temperature for 20 minutes, 10 µl of antibody or
isotype control antibody was added (PE-conjugated anti-CCR5 F(ab')2 182, R&D Systems; PE-conjugated IgG2a, BD Pharmingen) and cells were left at 4°C for 40 minutes. Antibodies were washed out twice in 2 ml PBS/1% FCS and once in 2 ml PBS, before fixing in 2% formol saline and analysing on a FACScan flow cytometer.

A dot plot showing the forward and side scatter thresholds (FSC and SSC respectively) and a histogram plot of the FL2-H axis was drawn before the lyophilised pellet of calibration beads was resuspended in 500 μl PBS/0.1% sodium azide and running through the FACS machine. Gates were adjusted around the bead singlet observed on the dot plot (Fig. 2.1A), and markers adjusted over the four peaks on the histogram plot to calculate the mean fluorescence of each peak (Fig. 2.1B). The lot-specific PE/bead values and the geometric mean of each histogram peak given in the histogram statistics (Fig. 2.1C) were entered into the Quantitative Calibration option in the CellQuest software. The software performs regression analysis and displays the slope, intercept and the correlation coefficient. These values were acquired by the QuantiQuest programme and used to calculate the number of antibodies bound per cell from the geometric mean value of histogram plots of subsequently acquired sample populations.

2.3.6.3 CCR5 down-modulation

Purified and activated PBMC were counted, and 1 x 10⁵ cells were used per sample. Cells were pelleted by centrifugation, and resuspended in 50 μl medium alone (for isotype control), or CCR5 inhibitor/RANTES at 25, 50 and 100 nM. Samples were incubated at 37°C for 0 or 90 minutes and cooled to 4°C on ice, to prevent recycling of CCR5 to the cell surface, before the inhibitors were washed out by centrifugation in 1 ml cold PBS/1% FCS/0.1% azide. Supernatant was removed and cells resuspended in 50 μl anti-CCR5 primary antibody (MAB 182, R&D Systems) or the isotype control (IgG2B, Dako) at 5 μg/ml. After incubation on ice for 45 minutes, cells were washed twice with cold PBS/1 FCS/0.1% azide and resuspended in 50 μl of a goat anti-mouse FITC-conjugated antibody (Dako) diluted 1:40 in PBS/1% FCS/0.1% azide. Following a further 45 minute incubation on ice, cells were washed once with 1 ml cold PBS/1% FCS/0.1% azide and once with 1 ml cold PBS/0.1% azide before resuspending in 500 μl cold 2% formol saline. Cells were analysed on a FACScan (BD Pharmingen) using the CellQuest software package. From the histogram statistics, the relative CCR5 surface
expression was calculated as 100 x (mean channel fluorescence [stimulated] - mean channel fluorescence [isotype control])/(mean channel fluorescence [medium alone] - mean channel fluorescence [isotype control]).

2.3.7 Immunofluorescent microscopy

Glass coverslips of 13 mm diameter were placed in the wells of a 24-well plate and sterilised by washing in 70% ethanol. After rinsing in sterile PBS, cells were plated at the appropriate density (primary astrocytes at 1 x 10^5/ml; cell lines at 4 x 10^4/ml) and left overnight at 37°C to adhere. Cells being stained for the intracellular marker GFAP were washed once in PBS before fixing in a cold (-40°C) 1:1 methanol:acetone mix for 5 - 10 minutes. Cells being stained for membrane-bound proteins were fixed following immunostaining. Cells were gently rinsed in PBS before incubating for one hour with 200 µl of the primary antibody. Antibody was removed by gently rinsing twice in PBS/1% FCS and antigen was detected by incubating for one hour in 200 µl of the secondary antibody (goat anti-mouse FITC-conjugate or swine F(ab')2 anti-rabbit FITC, diluted 1:40 or 1:15 respectively; Dako USA). Cells were gently rinsed once in PBS/1% FCS, twice in PBS and covered in 2% formol saline. All immunostained cells were analysed on a fluorescence microscope, using the IPLab software (Scanalytics, Inc.).

2.3.8 Transfection of adherent mammalian cells

2.3.8.1 Calcium phosphate transfection of mammalian cells

Cells were plated the day before transfection, at a density dependent upon the cell type and the rate of division, to allow a 40 - 70% confluence on the day of transfection. Growth medium was replaced three hours before the transfection. All calcium phosphate transfection reagents (Gibco Invitrogen Corporation) were thawed and individual components mixed well before making up the required solutions. Table 2.6 shows quantities used. Calcium chloride was mixed with the DNA and appropriate volume of MilliQ water in one sterile tube, and the equivalent volume of 2x Hepes-Buffered Saline (HBS) was placed in another. The DNA-calcium mix was added drop-wise to the constantly but gently agitated HBS. After 30 minutes incubation at room temperature, the co-precipitated DNA:calcium mix was added evenly over the plate whilst swirling in order to maintain a constant pH and to evenly disperse the precipitate. After 6 hours, the transfection media was replaced and cells gently rinsed twice before
the addition of fresh medium. Cells that had been transfected with HIV coreceptors were analysed for expression by infection with a suitable HIV strain 48 hours after transfection. Cells transfected with viral DNA were analysed as described in section 2.4.2.3.

<table>
<thead>
<tr>
<th>Per well of 6-well plate</th>
<th>Per 10cm² dish</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>3 µg</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>10.34 µl</td>
</tr>
<tr>
<td>Sterile H₂O</td>
<td>To 83.4 µl</td>
</tr>
<tr>
<td>10x HBS</td>
<td>83.4 µl</td>
</tr>
</tbody>
</table>

Table 2.6. Calcium phosphate transfection. Quantities and volumes of components used for calcium phosphate transfection in different sized culture plates.

2.3.8.2 Transfection by FuGENE 6 transfection reagent

Cells were plated in a 6-well plate the day before transfection at 1 x 10⁵ cells/ml in serum-free, antibiotic-free media. Maximum efficiency of transfection was achieved with a FuGENE:DNA ratio of 3:1. In a sterile tube, serum-free medium was added to make the total reaction volume up to 100 µl, before adding 9 µl FuGENE (taking care not to touch the plastic tube) and 3 µg DNA. The reaction mix was left at room temperature for 15-45 minutes before being added dropwise to the cells, swirling to ensure even distribution. Cells were left overnight before medium was replaced with serum-containing medium. Protein expression was determined 48 - 72 hours post-transfection, and stable cells were selected as described below.

2.3.9 Selection of stable transfectants

Highly expressing cells were cloned from the bulk transfected population by limiting dilution. Cells were removed with trypsin, resuspended at 1 x 10⁵/ml and diluted in ten-fold dilutions down to 1 x 10²/ml, in 20 ml medium containing the appropriate antibiotic for selection (e.g. puromycin, G418; see Table 2.3). 5 ml of each dilution was added to 11 ml medium to make half-log dilutions of each cell concentration. These eight cell concentrations were plated in flat-bottomed 96-well plates in 100 µl volumes and left in a humidified atmosphere with 5% CO₂ at 37°C. Cells were fed every 3-4 days with 25 µl of growth medium with 10% FCS and wells were checked for growth of a single cell colony. When colonies became confluent they
were transferred to a 48-well plate and gradually bulked up until a T25 flask was confluent. Receptor expression was determined by flow cytometry.

2.4 Virus Infectivity Assays

2.4.1 Virus strains

Well characterised virus strains used in this thesis are described below in Table 2.7. All HIV-1 isolates are group M, subtype B unless denoted otherwise. Chapter 5 of this thesis investigates the alternative coreceptor properties of a large panel of poorly characterised subtype B and C isolates. Details of these 34 isolates, including country of origin and patient data, are outlined below in Table 2.8. All isolates were obtained through the NIH AIDS Research and Reference Reagent Program, except where noted. The coreceptor properties of these isolates are described in detail in Tables 5.1, 5.2 and 5.3 and 5.4.

2.4.2 Production of virus stocks

2.4.2.1 Production of primary virus stocks

Primary virus isolates were propagated in PHA and IL-2-activated PBMCs (section 2.3.5.1). 5 x 10^6 cells were incubated at 37°C for 3 hours with 0.5 - 1 ml of a seed stock of a primary virus strain. Cells were diluted by the addition of 4 ml RPMI/20% FCS/10% IL-2 and incubating at 37°C in an upright flask. Virus levels were measured daily by a radioactive RT assay (section 2.4.5) from day 3 and harvested when virus production peaked. Harvesting was done by pelleting PBMCs at 325 g for 5 minutes, removing supernatant and aliquoting 0.5 ml volumes into cryovials (Alpha Inc.). Virus was snap-frozen in liquid nitrogen before being transferred to gas-phase liquid nitrogen for long-term storage.
<table>
<thead>
<tr>
<th>Virus</th>
<th>Strain</th>
<th>Isolate type</th>
<th>R5/X4</th>
<th>Other Coreceptors</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-1</td>
<td>SF162</td>
<td>Primary; MC</td>
<td>R5</td>
<td>R3, R8</td>
<td>M65024 (Cheng-Mayer et al., 1989)</td>
</tr>
<tr>
<td></td>
<td>E80</td>
<td>Primary</td>
<td>R5</td>
<td>-</td>
<td>(Simmons et al., 1996)</td>
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<tr>
<td></td>
<td>2076</td>
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<td>R3, R8, CXCR6, GPR15</td>
<td>(Simmons et al., 1996)</td>
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<td></td>
<td>89.6</td>
<td>Primary; MC</td>
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<td>APJ</td>
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<td>2044</td>
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<td>GUN-1WT</td>
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<td>R5X4</td>
<td>R3, R8,(GPR1)</td>
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<td>GUN-1v</td>
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<td>(R3)(R8)(CXCR6)(GPR15)</td>
<td>(Simmons et al., 1996)</td>
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<td>HAN-2</td>
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<td>P1019</td>
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<td>C3</td>
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<td>-</td>
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<td>RU570*</td>
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<td>-</td>
<td>(Bobkov et al., 1994)</td>
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<td>(R1)</td>
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<tr>
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<td>Rod B</td>
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<td>-</td>
<td>(Daniel et al., 1985)</td>
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<tr>
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<td>17E-fr</td>
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<td></td>
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<td>MC</td>
<td>R5X4</td>
<td>R2b, R5, GPR1</td>
<td>See above</td>
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</table>

Table 2.7. Virus isolates and coreceptor use. All virus isolates used within this study, and details of coreceptors able to support their entry. MC = molecular clone; TCLA = T cell-line adapted. Parentheses indicate a weak use of a coreceptor. * denotes a clade G isolate.
<table>
<thead>
<tr>
<th>Virus</th>
<th>Origin</th>
<th>Patient/Genbank Information</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>97USNG30</td>
<td>Nigeria</td>
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</tr>
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<td></td>
</tr>
<tr>
<td>*MJ4</td>
<td>Botswana</td>
<td>MC. Backbone from 23 yr M, CDC disease</td>
<td>(Ndung'u, Renjifo, and Essex, 2001)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>stage III. Envelope from 25 yr M, CDC</td>
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<tr>
<td></td>
<td></td>
<td>disease stage I. AF110959.</td>
<td></td>
</tr>
<tr>
<td>96ZM651</td>
<td>Zambia</td>
<td>AF286224</td>
<td></td>
</tr>
<tr>
<td>98TZ013</td>
<td>Tanzania</td>
<td>Taken ≤2 yrs post-seroconversion. AF286234</td>
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<tr>
<td>98TZ017</td>
<td>Tanzania</td>
<td>Taken ≤2 yrs post-seroconversion. AF286235</td>
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<tr>
<td>95IN21068</td>
<td>India</td>
<td>21 yr M. Isolated 1 yr post sero-conversion.</td>
<td>(Lole et al., 1999)</td>
</tr>
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<td></td>
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<td>AF411964</td>
<td>(Cilliers et al., 2003)</td>
</tr>
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<td>AF411967</td>
<td>(Cilliers et al., 2003)</td>
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<td>South Africa</td>
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<td>(Cilliers et al., 2003)</td>
</tr>
<tr>
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<td>South Africa</td>
<td>Unknown</td>
<td>(Cilliers et al., 2003)</td>
</tr>
<tr>
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<td>South Africa</td>
<td>Unknown</td>
<td></td>
</tr>
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**Table 2.8. HIV-1 subtype C isolates.** Details of the country of origin, patient information, relevant references and genbank accession codes (where sequence data is available) for a panel of 34 primary HIV-1 subtype C isolates. * indicates a molecular clone. F = female; M = male; MSM = men who have sex with men; CSW = sex with a commercial sex worker. * denotes isolates gratefully received from Dr. Lynne Morris.
2.4.2.2 Production of TCLA virus stocks

T-cell line adapted (TCLA) virus isolates were propagated in the CD4-positive cell line Molt-4 Clone 8 (Table 2.4). Cells were counted and 2 x 10^6 cells were pelleted by centrifuging for 5 minutes at 1500 rpm before being resuspended in 0.5 - 1 ml virus. Cells were incubated at 37°C for 3 hours before 3 - 3.5 ml media was added and cells incubated in an upright flask at 37°C. When large syncytia were observed, virus supernatant was harvested by centrifuging the cell suspension at 210 g for 5 minutes, aliquoting and freezing as described in section 2.4.2.1. The remaining cells were co-cultivated with 5 x 10^6 fresh uninfected cells in 1 ml media and incubated at 37°C for 3 hours, before making up to 15 ml total volume and incubating further at 37°C. The culture was followed until syncytia were observed again, and virus was harvested as described.

2.4.2.3 Production of virus stocks from DNA

Plasmids containing full-length infectious viruses, such as SIV 17E-fr, SIV 17E-fr and SIVmac239, were transfected into 293T cells by calcium phosphate transfection as described in section 2.3.8. Following transfection, supernatant was removed daily to determine virus production by radioactive RT assay or RT ELISA (sections 2.4.5.1 and 2.4.5.4). Adherent 293T cells were then co-cultivated with PBMCs at 0.8 x 10^6/ml. Virus production was measured daily by radioactive RT assay and supernatant harvested when viral production peaked.

2.4.3 Production of adenovirus vectors

2.4.3.1 Amplification of adenovirus

The adenovirus-CD4 vector DNA was gratefully received from Koichi Miyake at the Nippon Medical School, Tokyo (Yasukawa et al., 1997). It contains the human CD4 gene in place of the early adenovirus gene region E1, under the control of a CAG promoter (cytomegalovirus enhancer with a chicken β-actin promoter). The adenovirus-CCR5 vector was a generous gift from Jacqueline Reeves at the University of Pennsylvania. This vector is a recombinant clone formed by cotransformation of the E. coli strain (BJ5183) with an adenovirus shuttle vector (pAdTrack-CMV), encoding both CCR5 and GFP under the control of a cytomegalovirus (CMV) promoter, with an ΔE1ΔE3 adenoviral backbone plasmid (pAdEasy-1)(He et al., 1998). The adenovirus vectors were transfected as previously described into 70% confluent adenovirus-
complementing 293 cells, a cell line initially transformed with sheared adenovirus DNA, which results in the adenovirus vector being complemented in trans by the cell-produced E1 gene products to produce an infectious, replication-defective adenovirus particle. When cells detached (3-4 days), adenovirus particles were harvested by centrifuging, removing medium and resuspending cells in 10 ml fresh medium. Cells were lysed by snap freezing and thawing three times before broken cells and debris were removed by centrifugation. Supernatants were aliquoted in cryovials and frozen as described in section 2.3.3.

### 2.4.3.2 Determination of adenovirus titre by plaque assay

The titre of adenovirus stocks was determined by plaque-forming assay. 293 cells were plated in 1 ml at 1 x 10^5/ml, in 12-well plates. The following day, media was replaced with 500 µl of serum-free DMEM. Adenovirus stocks were diluted 10-fold from undiluted to 1 x 10^10, and 1 µl of each dilution added to a single well of the 12-well plate before cells were incubated for 60 minutes at 37°C. A 5% Seaplaque® agarose solution, dissolved in sterile PBS, was melted in boiling water on a hot-plate before it was diluted 1:4 in DMEM/5% FCS. Virus was removed, and the cell monolayer was gently covered in 1 ml agarose/DMEM mix. After allowing to solidify at room temperature for 10 minutes, plates were transferred to a humidified incubator at 37°C where they were left until plaques of cell death were observed (approximately 8-12 days). Presence of adeno-CCR5 at the sites of plaques was confirmed by the visualisation of GFP by fluorescent microscopy (Fig. 2.2A). CCR5 expression levels were determined by flow cytometric analysis of adeno-CCR5 infected and uninfected U87/CD4 cells (Fig. 2.2B). Due to the high levels of GFP produced by the ad-CCR5 vectors an allophycocyanin (APC)-conjugated CCR5 mab (BD Pharmingen) was used. Levels of CD4 expression following adenovirus infection was also measured by flow cytometry, with the CD4 mab Q4120 and the appropriate isotype control (IgG1)(Fig. 2.2C).
Figure 2.2. Adenovirus-mediated cell surface receptor expression. U87/CD4 cells were treated or untreated with Adeno-CCR5. Successfully transduced cells were detected by GFP fluorescence after 24 hours (A). Cell surface expression of CCR5 was confirmed by flow cytometry (B). CD4-expression levels on NP2 cells was determined by flow cytometry 24 hours post-infection (C).
2.4.4 HIV infection

2.4.4.1 Infection of adherent cell lines

Cells were plated the day before use in a 48-well tray at $3 \times 10^4$ cells/ml (NP2 cell line) or $2 \times 10^4$ cells/ml (U87 cell line). On the day of infection, media was removed from the wells, and cells infected with 100 µl of either ten-fold serially diluted virus (to determine titre), or with a specific virus dilution (to infect the desired number of cells). After incubating for 3 hours, cells were gently washed with growth medium and incubated for 72 hours in 500 µl of fresh medium. Infected cells were detected by the methods described in section 2.4.5.

2.4.4.2 Infection of PBMCs

Activated PBMCs were counted, and $1 \times 10^5$ cells plated in 100 µl of RPMI/20% FCS/10% IL-2 in V-bottomed 96-well plates. After centrifugation for 5 minutes at 210 g, media was removed and the cell pellet resuspended in 100 µl of virus at the appropriate concentration before incubating for 3 hours at 37°C. Virus was removed by washing cells 3 times by centrifugation for 5 minutes at 210 g in 100 µl RPMI/20% FCS/10% IL-2. Productive infection was monitored by removing 80 µl of cell-free supernatant and replacing with fresh medium on days 3, 6 and 9 of the infection. Virus levels were measured by p24 ELISA (section 2.4.5.2). All samples were done in triplicate.

2.4.4.3 Infection of macrophages

The day before they were required, macrophages were washed twice in 0.02% versene (Gibco Invitrogen Corporation, UK) and incubated in 10 ml of versene at 37°C to dislodge cells. Macrophages were removed gently with a cell scraper, centrifuged at 300 g for 7 minutes and resuspended in DMEM/10% human serum at $1 \times 10^5$/ml. Cells were then plated in 48-well plates, 500 µl per well, and left overnight to adhere. On the day of infection, media was removed from each well and 100 µl of virus at the appropriate dilution was added. Following incubation for 3 hours at 37°C, macrophages were rinsed once in DMEM/10% human serum and covered in 500 µl of the same media. Cells were then incubated at 37°C for 20 days, with supernatant removed every three days to monitor p24 production by p24 ELISA. Macrophages were also immunostained in situ for p24 on day 20 of infection (section 2.4.5.3).
2.4.4.4 Infection of primary astrocytes

Primary astrocytes were dislodged in trypsin:versene and plated in 48-well plates at 6 x 10⁴/ml (control cell lines were plated at 2 x 10⁴/ml). The following day media was removed and astrocytes were infected with either 100 μl of the adenovirus vector at a dilution conferring maximum infection of NP2/CCR5 cells with SF162, or 100 μl growth medium. After 3 hours virus was removed and cells rinsed once in media before incubating at 37°C in 500 μl DMEM/10% FCS. The next day, media was removed and cells were incubated for 3 hours with 100 μl of HIV/SIV at the appropriate dilution, before rinsing out and replacing with 500 μl fresh media. After 3 days, infected cells were detected by p24 immunostaining.

2.4.4.5 Infection of primary Leydig cells

Primary Leydig cells were prepared as described in section 2.3.5.4. When received, transport medium was removed and cells allowed to stabilise at 37°C in fresh medium. Cells to be plated were dislodged from the flask with a cell scraper, counted, and plated at 3 x 10⁴ in a 48-well plate. The following day, cells were either infected with adenovirus and subsequently with HIV as for astrocyte infection (see above), or were incubated with 100 μl of virus at the appropriate dilution for 6 hours at 37°C. Following incubation, 400 μl of media was added, and plates were left for up to 18 days. Supernatant was removed on day zero and every subsequent three days for virus production analysis by RT ELISA (section 2.4.5.4). Infected cells were also detected by p24 immunostaining (section 2.4.5.2).

2.4.5 Measurement of virus production

2.4.5.1 Radioactive reverse transcriptase assay

Supernatant was taken from an infected cell culture and centrifuged at 250 g for 5 minutes in a sealed 96-well plate. 10 μl of cell-free supernatant was added to 40 μl of radioactive reaction premix containing 5 μM Tris pH7.8, 4 μM KCl, 5 mM MgCl₂, 2 mM Triton-X 100, 2 mM dithiothreitol, 10 mM poly (rA) p(dT) (Pharmacia Biotech), 4 mM [methyl-³²H] thymidine 5'-triphosphate in ddH₂O per reaction. This mix was incubated for 3 hours at 37°C before harvesting onto a filter mat (Wallac) using a Sakron Combi cell harvester. The filter mat was washed in 2x SSC and dried on a hot plate before melting on a wax scintillator sheet (Perkin Elmer). Samples were read using a Wallac Micro Beta workstation. All samples were measured in duplicate, with a
chronically infected cell line as a positive control and uninfected PBMC cell-free supernatant as a negative control.

2.4.5.2 p24 ELISA

High-bind white plates (Nunc MaxiSorp) were coated with 100 μl of a p24 polyclonal antibody (D7320, Aalto Bioreagents) diluted to 5 μg/ml in 100 mM NaHCO₃ the day before use. After leaving overnight at room temperature, plates were washed twice with 200 μl of 1x TBS per well. Non-specific protein binding was blocked by incubating at room temperature for 30 minutes in 200 μl of 2% milk in TBS (dried skimmed milk, Marvel). A p24 standard (ADP620, MRC ADP reagents) was prepared by adding 1 μl of a 10 μg/ml stock to 999 μl TBS/0.05% empigen (benzalkonium chloride, Surfachem Ltd) and diluting 1 in 3.2, 1 in 9.6, 1 in 30.7, 1 in 92.16, 1 in 294.9 and 1 in 884.7 to make a standard curve with values of 10000, 3000, 1000, 300, 100 and 30 pg/ml p24 respectively. Sample supernatants were prepared by adding 80 μl of cell-free supernatant to 20 μl of 5% empigen in TBS and further diluting 1 in 20 in TBS. If samples were likely to have high virus levels a further 1 in 10 dilution (1 in 200 total) was done in TBS/0.05% empigen to maintain a constant concentration. Plates were washed once with TBS before adding 100 μl of the 6 standards, diluted samples plus a negative control (0.05% empigen). Plates were covered and incubated at room temperature for 4 hours. After washing twice with 200 μl per well of TBS, 100 μl of the secondary antibody mix was added per well, containing a 1:2000 dilution (0.5 μg/ml) of a second anti-p24 antibody directly conjugated to alkaline phosphatase (EH12E1-AP, MRC ADP452) in 4% milk powder, 20% sheep serum (Gibco Invitrogen Corporation Brl, UK), 0.5% Tween 20 (Sigma) in TBS. After a one-hour incubation at room temperature plates were washed five times in TBS/0.5% Tween20, incubated in the dark for one hour with 100 μl of AP substrate (Lumiphos, UK) at room temperature and AP was detected using an Anthos Lucy 1.0 luminometer. All samples, standards and controls were run in duplicate.

2.4.5.3 p24 immunostaining

Infected cells were detected by removing media from each well, rinsing cells in PBS and fixing in a cold (-40°C) 1:1 methanol-acetone mix. After 5-10 minutes cells were rinsed again with PBS/1% FCS/0.1% azide and 100 μl of a p24 polyclonal antibody was added. For HIV-1, the anti-p24 was a 1:1 mix of 38:96K and EF7 from
the AIDS Reagent Program (365 and 366 respectively) and HIV-2 was detected using a mix of 6 HIV-2\(^+\) patient serum samples (WHO panel C; MRC ARP) diluted 1:40 or 1:4000 in PBS/1% FCS, respectively. Following an hour-long incubation, cells were rinsed twice with PBS/1% FCS/0.1% azide before incubating with 100 µl of the secondary antibody (goat anti-mouse or goat anti-human β-galactosidase conjugate for HIV-1 and HIV-2 respectively; Southern Biotechnology Associates, Inc) diluted in PBS/1% FCS/0.1% azide at 1:400 for one hour. Cells were then washed twice in PBS/1% FCS/0.1% azide and once in PBS/0.1% azide before adding 500 µl of the substrate (PBS with 3 mM potassium ferricyanide, 3 mM potassium ferrocyanide, 1 mM magnesium chloride and 0.5 mg/ml X-gal) and incubating for 1-4 hours at 37°C. Infected cells were counted under visible light microscopy and the average number of focus-forming units (FFUs)/ml was determined.

2.4.5.4 Reverse transcriptase ELISA

Presence of RT in cell supernatant was determined and quantified using a high sensitivity, non-radioactive RT ELISA kit (CavidiTech, Uppsala, Sweden), according to manufacturers' instructions. The kit uses an immobilised template/primer construct that is elongated by the RT present in the sample. As DNA is synthesised it incorporates bromo-deoxyuridine triphosphate (BrdUTP) from the reaction mix, which is later detected by the RT product tracer, an alkaline phosphatase-conjugated anti-BrdUTP antibody. Alkaline phosphatase activity is proportional to the sample RT activity, and is measured colourimetrically using an Anthos Lucy 1.0 luminometer following conversion of the dNPP substrate to a dephosphorylated dNP.

2.4.6 Inhibition assays

2.4.6.1 Small molecule inhibitors of CCR5

The small molecule inhibitors of CCR5 developed by Pfizer Global Research and Development arrived in powder form and were reconstituted in sterile DMSO to a 1 mM concentration. Compounds were further diluted to 20 µM in DMEM/5% FCS and stored at -80°C in 1 ml aliquots until required. Table 2.9 shows the activity of each compound with respect to inhibition of CCR5 ligand binding, gp120 binding and HIV infection.
Table 2.9. Small molecule inhibitors of CCR5. CCR5 ligand-binding, gp120 binding and infection inhibition data for six CCR5 small molecule inhibitors developed by Pfizer Global Research and Development. All experiments contributing to this table were carried out at Pfizer GRD, Sandwich, Kent.

<table>
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<tr>
<th>Compound</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
<th>M&lt;sub&gt;r&lt;/sub&gt;</th>
<th>MIP-1β</th>
<th>gp120</th>
<th>Antiviral</th>
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<td>&lt;0.1</td>
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<td>4</td>
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<td>608.76</td>
<td>&gt;1000</td>
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<td>526.14</td>
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2.4.6.2 Other HIV coreceptor inhibitors

RANTES was purchased from Peprotech Inc (Rocky Hill, New Jersey). TAK-779 was obtained through the AIDS Research and Reference Reagent Program, NIAID, NIH (Baba et al., 1999). AMD3100, a small molecule inhibitor of CXCR4, was a generous gift from Drs. Erik De Clercq and Dominique Schols. The ligands eotaxin (CCR3) and 1309 (CCR8) were purchased from R&D Systems, as were the chemokine homologues vMIP-I and vMIP-II. All inhibitors were diluted to a concentration of 80 μg/ml (10 mM) in sterile water, aliquotted and stored at -80°C until needed. The peptide apelin-36, the ligand for APJ, was purchased from the Peptide Institute Inc (Japan) and reconstituted in sterile water.

2.4.6.3 Inhibition of adherent cell line infection

Cells were seeded in a 48-well tray as described in section 2.4.4. The next day, media was removed and cells were incubated for 1 hour with 75 μl of inhibitor at 2x final concentration, diluted in the selection medium appropriate for the cell line being used. Control wells had 75 μl of growth medium added. Following pre-incubation, 100 FFU/ml of virus was added in 75 μl and plates were incubated for a further 3 hours. Cells were then rinsed twice in media and 500 nM inhibitor was replaced at 1x concentration. Cells were grown in a humidified atmosphere for three days before infected cells were detected by p24 immunostaining and counted. All samples were done in duplicate.
2.4.6.4 Inhibition of PBMC infection

Activated PBMCs were counted, and $1 \times 10^5$ cells plated in V-bottomed 96-well plates in 100 µl of RPMI/20% FCS/10% IL-2. After centrifuging for 5 minutes at 210 g, media was removed and cells were resuspended in 50 µl of 2x inhibitor. Following incubation for 1 hour at 37°C, 50 µl of virus at the appropriate concentration was added, mixed and cells were incubated at 37°C for 3 hours. After incubation, cells were washed three times in 200 µl medium by centrifuging for 5 minutes at 210 g. Cells were resuspended in 150 µl inhibitor at 1x concentration and left at 37°C. Cell-free supernatant was removed on day 0, 3, 6 and 9 post-infection, and virus production measured by p24 ELISA. Inhibitor was replenished upon each removal of supernatant. All samples were done in triplicate.

2.4.6.5 Inhibition of macrophage infection

Macrophages were detached by incubation in versene for 10 minutes at 37°C, counted then seeded in a 48-well plate at $1 \times 10^5$ cells per well. Inhibition and subsequent infection was carried out as described in section 2.4.6.2 for adherent cells. Infectivity of viruses with low titres on macrophages was enhanced by spinoculation (O'Doherty, Swiggard, and Malim, 2000). Following 1 hour preincubation with inhibitor, virus was added and plates were sealed. Plates were spun at 250 g at room temperature for 45 minutes, before transferring to a 37°C incubator for the remaining 2 hours 15 minutes. Virus was then washed out, and cells incubated for up to 20 days. Supernatant was harvested and replaced as for PBMC assay (section 2.4.5.3) on days 4, 8, 12, 16 and 20 and virus levels measured by p24 ELISA. Macrophages were also immunostained in situ on day 20 for intracellular p24.

2.4.6.6 Inhibition of astrocyte infection

Astrocytes were seeded in 48-well plates at $6 \times 10^4$/ml and incubated at 37°C overnight to adhere before infecting with adenovirus as described in section 2.4.4.4. The day after adenovirus infection, astrocytes were preincubated for one hour at 37°C with 75 µl of the inhibitor at 2x the required concentration. After removal of the medium, 75 µl of undiluted virus was added and cells left to infect for three hours. After the addition of 350 µl of medium, cells were incubated for 7 days at 37°C before infected cells were detected by p24 immunostaining and RT ELISA.
Inhibition of Leydig cell infection

Leydig cells were gently dislodged with a cell scraper, counted, and seeded in a 96-well plate at $3 \times 10^4$/ml in 200 µl. After allowing to adhere overnight at 37°C, cells were incubated for 1 hour in 30 µl of inhibitor at 2x concentration before 30 µl of virus was added. Cells were left to infect overnight before supernatants were removed and 100 µl of inhibitor was replaced at 1x concentration. Cells were left at 37°C for up to 18 days, with supernatant being removed for RT ELISA analysis every 3 days. Inhibitor was replenished following supernatant harvest.
CHAPTER 3

Inhibition of CCR5-mediated infection by novel small molecule inhibitors of CCR5

3.1 Introduction

Current anti-retroviral therapy (HAART) comprises of several drug strategies, with varying combinations of nucleoside reverse transcriptase inhibitors (NRTIs e.g. zidovudine), non-nucleoside reverse transcriptase inhibitors (NNRTIs e.g. nevirapine) and PR inhibitors (PIs e.g. indinavir)(section 1.8.2)(Yeni et al., 2002). These regimens are highly effective at reducing viral loads, partially restoring the immune system and increasing the life span of infected individuals (Gulick et al., 1997; Hammer et al., 1997; Kaufmann et al., 2000; Palella et al., 1998). However, the complex regime involving a range of drugs to be taken at varying time-points makes adherence to therapy difficult (Tsasis, 2001). Anti-retrovirals also have many detrimental side effects, including hepatotoxicity, (nevirapine), teratogenicity (efavirenz), psychoactivity (efavirenz) and lipid abnormalities such as increases in triglyceride and low-density lipopolyprotein levels (ritinovir)(Table 1.5)(Yeni et al., 2002). These factors, in combination with the frequent evolution of intra-class resistance to inhibitors reflect the urgent need for new drug targets (Brenner, Turner, and Wainberg, 2002; De Clercq, 2002).

Molecules that target HIV entry are the subject of much interest. Whilst RT and PR inhibitors are effective against actively replicating virus, they leave a viral reservoir in the form of proviruses in latently infected cells (section 1.8.2)(Blankson, Persaud, and Siliciano, 2002; Pomerantz, 2002). An entry or fusion inhibitor used in combination with RT or PR antiretrovirals could prevent failure of current therapeutic regimens, due to incomplete adherence and/or evolution of resistance. Of the two major HIV coreceptors, CCR5 plays an essential role in virus transmission, demonstrated by the evidence that individuals homozygous for a 32-base pair deletion in this chemokine receptor are largely resistant to infection (Dean et al., 1996; Huang et al., 1996; Liu et al., 1996; Samson et al., 1996). The fact that virus isolates exploiting CCR5 persist throughout infection, even in late stages of disease (de Roda Husman et al., 1999; van Rij et al., 2002), and that Δ32/Δ32 CCR5 homozygous individuals remain in good
health despite loss of CCR5 function (Samson et al., 1996) makes CCR5 an ideal candidate for novel therapeutic strategies.

Although CCR5 ligands RANTES and MIP-1β, and particularly modified analogues such as AOP-RANTES, are effective at inhibiting CCR5-mediated infection in vitro they carry the risk of potential adverse inflammatory responses (Cocchi et al., 1995; Deng et al., 1996; Dragic et al., 1996; Oravec, Pall, and Norcross, 1996; Simmons et al., 1997). Moreover, the cost of producing small proteins, and the requirement of intravenous administration, makes widespread use of chemokine derivatives prohibitive. These factors favour small organic molecules as the preferred form of coreceptor inhibitor. Small molecule inhibitors that bind CCR5 or CXCR4 have already been described previously (Baba et al., 1999; De Clercq et al., 1994; Donzella et al., 1998; Schols et al., 1997a; Schols et al., 1997b; Strizki et al., 2001), and have proven to be extremely effective inhibitors of infection in vitro and in vivo.

The anilide derivative TAK-779, and the piperidin-butane and pyrrolidine compounds by Merck, inhibit CCR5-mediated HIV infection by binding to a small pocket within the transmembrane helices of CCR5 (1, 3, 5 and 7 for TAK-779, and 2, 3, 6 and 7 for the Merck inhibitors)(Fig. 1.13 and section 1.8.3.1)(Castonguay et al., 2003; Dragic et al., 2000). In addition, TAK-779 inhibits SIV infection via CCR2b (Dragic et al., 2000). A second CCR5 inhibitor, SCH-C, also exhibits potent anti-HIV activity both in vitro and in a SCID-hu mouse model (Strizki et al., 2001). A third small molecule inhibitor of CCR5, UK-427857, possesses a broad cross-clade activity at extremely low doses, both in vitro and in vivo (Abel et al., 2003; Napier et al., 2003). The low toxicity and oral bioavailability of these inhibitors indicate that such molecules would be a viable addition to current therapies (Abel et al., 2003; Strizki et al., 2001).

This chapter describes the analysis of a series of six heterocycle-substituted azabicycloalkane molecules from the same library of compounds as UK-427857, discovered and synthesised at Pfizer Global Research and Development (Dorr et al., 2003). The generic structure of this class of inhibitor is shown in Fig. 3.1A. The capacity of all six molecules (UK-396794-27, UK-383990, UK-387323-03, UK-399371-03, UK-400343-03 and UK-403341-03; UK patent number WO/0038680) to inhibit CCR5-mediated infection of cell lines by R5 isolates of HIV-1 was determined. Subsequently, the two most potent inhibitors, in addition to the least potent inhibitor,
were analysed for their ability to inhibit a range of R5 and R5X4 isolates of HIV-1, HIV-2 and SIV. Their effect on a range of cell types, including primary PBMCs and macrophages, the major cellular targets of HIV-1 in vivo, is described.
3.2 Results

3.2.1 Small molecule antagonists of CCR5 inhibit infection by HIV

The capacity of the six CCR5-specific small molecule inhibitors to block infection by two R5-tropic HIV-1 strains (SF162, derived from a molecular clone, and E80, a primary isolate) was evaluated. Compounds UK-396794-27 and UK-400343-03 were the most efficient inhibitors against both SF162 and E80 (Fig. 3.1B).

![Diagram of the generic structure of the class of heterocycle-substituted azabicycloalkane inhibitors of CCR5, developed at Pfizer Global Research and Development (A). U87/CD4/CCR5 was pre-treated with 0, 10, 100 and 1000 nM of the CCR5 inhibitors for 1 hour before infecting with the R5 HIV-1 isolates SF162 and E80 (B). Infected cells were detected by immunostaining for viral antigens. 100%-infection was calculated for each virus independently and represents the mean number of infected cells in the absence of inhibitors. All samples were done in duplicate, and error bars represent the standard deviation of the mean. Data is representative of at least three independent assays.](image-url)
UK-397694-27 reduced infection of both isolates by 100% at 50 nM, whilst UK-400343-03 inhibited E80 by 100% and SF162 97%, at the same concentration. Compounds UK-383990 and UK-403341-03 were the least effective, inhibiting SF162 infection by 75% and 94% respectively, and E80 by 50% and 60% respectively, at 500 nM. Compounds UK-387323-01 and UK-399371-03 were intermediate in their capacity as HIV-1 inhibitors (Fig. 3.1B). The order of efficiency of inhibition by the six compounds was consistent on both virus isolates. As a result of this, further in-depth studies on these inhibitors were only carried out with the two most effective (UK-396794-27 and UK-400343-03) and one of the least effective (UK-403341-03) inhibitors in this series.

3.2.2 Anti-HIV small molecules inhibit CCR5- and CCR2b-mediated infection

In addition to inhibiting CCR5-mediated infection by HIV, the CCR5 inhibitor TAK-779 blocks infection via the closely related chemokine receptor CCR2b (Dragic et al., 2000). As both TAK-779 and this series of inhibitors have similar molecular weights and possess a basic core, they may occupy a similar space in CCR5 (P. Dorr, personal communication)(Shiraishi et al., 2000). The coreceptor specificity of one of the three small molecule inhibitors of CCR5 was therefore tested. Indicator cell lines stably expressing CD4 and a range of coreceptors were pre-treated with compound UK-400343-03 or a natural chemokine ligand/small molecule inhibitor that binds the particular coreceptor under study (Fig. 3.2). Treated and untreated cells were then infected with an HIV or SIV isolate, depending upon coreceptor usage (Table 2.7). The HIV-2 strain TER was used for infection via CCR5, CCR8 and GPR1; HIV-1 strain GUN-1v for CCR2b; HIV-1 strain 2044 for CXCR4; HIV-1 HAN-2 for CCR3; HIV-1 89.6 for APJ and SIVman4 for CXCR6.

On CCR5-expressing cells, compound UK-400343-03 was a more potent inhibitor than the CCR5 ligand RANTES, with 100% inhibition being observed at 500 nM in comparison to 85% inhibition with the same concentration of RANTES (Fig. 3.2). Although UK-400343-03 had no significant effect on infection via the coreceptors CCR3, CCR8, CXCR4, CXCR6, GPR1, or APJ, it did inhibit GUN-1v infection of U87/CD4/CCR2b cells. CCR2b-mediated infection by this isolate was completely inhibited by compound UK-400343-03 at 50 nM, in comparison to TAK-779, which gave 96% inhibition (Fig. 3.2).
Figure 3.2. UK-400343-03 inhibits CCR5- and CCR2b-mediated infection by HIV. Indicator cell lines stably expressing CD4 and a range of coreceptors were pre-incubated with compound UK-400343-03 or the natural ligand/synthetic inhibitor for each coreceptor tested before infection with HIV or SIV. The value of 100%-infection was calculated for each coreceptor cell line independently, and represents an average of the number of infected cells in the absence of inhibitors. This value was in the range of 75 – 250 focus-forming units (FFU) for each assay. The %-infection in the presence of inhibitors was calculated from the relevant 100% value. All samples were done in duplicate, and the error bars represent the standard error of the mean. Graphs are representative of at least two independent assays.
3.2.3 CCR5-specific small molecule inhibitors of HIV do not inhibit infection via receptor down-modulation

Chemokines and chemokine analogues, such as RANTES and AOP-RANTES, inhibit CCR5-mediated HIV infection by inducing receptor down-modulation (Mack et al., 1998). Thus the effect of the two most potent CCR5-specific small molecules on cell surface expression of CCR5 was determined. The observation that CCR5 expression levels were identical in samples incubated in the presence of RANTES, the small molecule inhibitors or no inhibitor indicates that binding of the monoclonal antibody (mab) used to detect CCR5 was not prevented by the inhibitors (Fig. 3.3A).

![Graph A](image1.png)

![Graph B](image2.png)

**Figure 3.3. Inhibition of HIV infection does not occur by ligand-induced receptor internalisation.** The effect of CCR5 inhibitors on mab binding to CCR5 on PBMCs was determined by comparing the % cell surface expression of CCR5 in the presence and absence of inhibitors RANTES, UK-396794-27 and UK-400343-03 at 100 nM (A). CCR5 internalisation was determined by incubating activated PBMCs with increasing concentrations of inhibitor for 90 minutes, and measuring cell surface CCR5 levels by quantitative flow cytometry (B).

PBMCs were incubated with UK-396794-27, UK-400343-03 (the two most potent CCR5 inhibitors) or RANTES. Following 90 minutes incubation with increasing concentrations of RANTES, CCR5 expression was successively reduced to 30% of levels in the absence of the chemokine (Fig. 3.3B), similar to previously published
reports (Mack et al., 1998). At the same concentration, the two most potent CCR5 inhibitors had no effect on the surface expression of CCR5. This infers that, despite their ability to inhibit gp120-binding, HIV infection and MIP-1β binding (Table 2.9), these small molecule inhibitors do not to induce receptor internalisation.

3.2.4 R5X4-tropic isolates of HIV-1 are inhibited more efficiently than R5-tropic isolates

Previous studies have indicated that virus isolates able to use CXCR4 in addition to CCR5 for entry are more susceptible to inhibition by CCR5-inhibitors (Kledal et al., 1997). The capacity of the small molecule inhibitors to block infection by a panel of R5 and R5X4 isolates of HIV-1 was therefore examined.

Figure 3.4. R5X4 HIV-1 isolates are inhibited more efficiently than R5 isolates. U87/CD4/CCR5 cells were pre-incubated with 0, 10, 100 or 1000 nM of UK-396794-27, UK-400343-03 or UK-403341-03 for one hour, before infecting with a range of R5 (SF162, RU570, E80, C3)(A) or R5X4 (2076, 2028, HAN-2, 89.6)(B) HIV-1 isolates. 100%-infection represents the average number of cells infected by each virus independently in the absence of inhibitor, and was used to calculate the % infection in the presence of inhibitor. 100%-infection was in the range of 50-250 FFU. Error bars represent the standard error of the mean of at least three independent assays.
All four R5 strains (SF162, RU570, E80 and C3) were completely inhibited by compound UK-400343-03 at 50 nM, and by compound UK-396794-27 at 500 nM (Fig. 3.4A). Compound UK-403341-03 completely inhibited SF162 infection at 500 nM, but only reduced RU570, E80 and C3 infection by no more than 60%. Infection via CCR5 by the four R5X4 isolates (2076, 2028, 89.6 and HAN-2) was more sensitive to inhibition by all three compounds. UK-396794-27 and UK-403341-03 were ten-fold more potent against all R5X4 strains, with 100% inhibition being observed at 5 nM (Fig. 3.4B). Three of the R5X4 isolates (2076, 2028 and 89.6) were completely inhibited by compound UK-403341-03 at 500 nM, while HAN-2 was reduced to 20% of infection levels seen in the absence of inhibitor.

3.2.5 UK-396794-27 is a more potent inhibitor of HIV-1 infection than RANTES or TAK-779

HIV infection mediated via CCR5 can be inhibited by the CCR5-binding chemokine RANTES, as well as small molecules targeted to this chemokine receptor, such as TAK-779. The relative efficiency of UK-396794-27 in comparison to RANTES and TAK-779 as inhibitors of CCR5 was determined. UK-396794-27 was consistently better than RANTES, giving 100% inhibition at 5 nM (R5X4 isolates) and 500 nM or less (R5 isolates)(Fig. 3.5).

Both UK-396794-27 and RANTES were more effective against R5X4-tropic isolates of HIV-1. Even at 500 nM, none of the R5 HIV-1 isolates tested were inhibited by more than 60%, whereas all R5X4 strains with the exception of HAN-2 were completely blocked at this concentration. Like UK-396794-27 and RANTES, TAK-779 was more effective against R5X4 isolates than R5, although the effect was less pronounced. Both R5X4 isolates tested were completely inhibited at 50 nM, one R5 strain (SF162) was completely inhibited at 500 nM and one reduced to 30% at the same concentration (RU570).
Figure 3.5. Novel CCR5 inhibitors are more efficient than RANTES and TAK-779. U87/CD4/CCR5 cells were pre-incubated with 0, 10, 100 or 1000 nM of UK-396794-27, RANTES or TAK-779 before infecting with HIV-1. Inhibition of two isolates from each category (R5 strains SF162 and RU570, and R5X4 strain 2028 and HAN-2) is shown for TAK-779. 100%-infection represents the average number of cells infected by each virus independently in the absence of inhibitor, and was used to calculate the %-infected cells in the presence of inhibitor. 100%-infection was in the range of 50-250 FFU depending on the assay. Error bars represent the standard error of the mean of at least two independent assays.

3.2.6 Small molecule inhibitors are able to block infection of HIV-2 and SIV isolates including SIVmac239, which preferentially uses CCR5 E2

HIV-2 and SIV strains commonly have a broader spectrum of coreceptor use than HIV-1 isolates (Clapham and McKnight, 2002; Reeves and Doms, 2002). The small molecule inhibitors were therefore tested against a panel of HIV-2 and SIV isolates with varied tropisms, including SIVmac239 that preferentially requires the E2 domain of CCR5 for entry (Edinger et al., 1997a). Overall, the HIV-2 strains were blocked less effectively than the HIV-1 strains, but the order of efficiency of the three
compounds remained the same (UK-400343-03>UK-396794-27>UK-403341-03)(Fig. 3.6). As seen with HIV-1, the R5X4-tropic HIV-2 isolate JAU was more sensitive to inhibition than the R5 isolate (Fig. 3.6A). SIVman4, a T-cell line adapted SIV strain that uses CXCR4 inefficiently, was inhibited to the same extent as the R5-tropic HIV-2 isolate ALI (Fig. 3.6B). The T-tropic SIVmac239 was blocked better than SIVman4 and as efficiently as the R5X4-tropic HIV-2 strain JAU (Fig. 3.6B). The preferential use of CCR5 E2 by SIVmac239 thus does not adversely affect sensitivity to CCR5 inhibitors.

**Figure 3.6. CCR5-specific small molecule inhibitors block infection by HIV-2 and SIV isolates.** U87/CD4/CCR5 cells were pre-incubated with 0, 10, 100 or 1000 nM of UK-396794-27, UK-400343-03 or UK-403341-03 for one hour. Cells were infected with the HIV-2 isolates ALI (R5), JAU (R5X4) and TER (R5 plus others)(A), or with two SIV strains, SIVman4 and SIVmac239 (B). 100%-infected cells represents the average number of cells infected by each virus independently in the absence of inhibitor, and was used to calculate %-infection in the presence of inhibitor. 100%-infection was in the range of 50-200 FFU. Error bars represent the standard error of the mean of at least two independent assays.
Figure 3.7A. The efficiency of the small molecule inhibitors of CCR5 is dependent upon cell type. NP2/CD4/CCR5 (A) and U87/CD4/CCR5 (B) cells were infected with two R5 (SF162 and E80) and two R5X4 (2076 and 89.6) HIV-1 isolates, following pre-incubation for one hour with 0, 10, 100 or 1000 nM of UK-396794-27, UK-400343-03 or UK-403341-03. 100% infection represents the average number of cells infected by each virus independently in the absence of inhibitor, and was used to calculate the % infected cells in the presence of inhibitor. 100% infection was in the range of 50-200 FFU. Error bars represent the standard error of the mean, and each graph is representative of at least three independent assays.
3.2.7 The antiviral potency of small molecule inhibitors is dependent upon cell type

The potency of the CCR5 inhibitors at blocking HIV infection of the cell line U87/CD4/CCR5 was compared to their potency on the cell line NP2/CD4/CCR5. Although both cell lines are astroglia-derived, infection of the NP2 cell line with both R5 and R5X4 isolates of HIV-1 was more resistant to inhibition by all three compounds, although their order of efficiency on both cell lines remained the same (Fig. 3.7A).

The relative expression level of CD4 on the two cell types was not significantly different (Fig. 3.7B); however, CCR5 expression levels were far greater on the NP2/CD4/CCR5 cells, possibly explaining the relative resistance of this cell line to inhibition. In order to determine if the differences in sensitivity to these CCR5 inhibitors was due to the higher cell surface levels of CCR5, or due to another cell-specific factor, an adenovirus vector containing CCR5 was used to increase CCR5 levels on the U87/CD4/CCR5 cell line. Infection of NP2/CD4 cells with ad-CCR5 was sufficient to confer susceptibility to infection with the R5-tropic HIV-1 SF162, confirming that adenovirus-expressed CCR5 is active as an HIV coreceptor (Fig. 3.7C).

U87/CD4/CCR5 cells treated with adeno-R5 (Fig. 3.7D, right panel) expressed higher levels of CCR5 than untreated cells (Fig. 3.7D, left panel), yet the small molecule inhibitors are equally as potent at inhibiting the R5X4 isolate 2076 on both. Compound UK-400343-03 completely inhibited infection of both ad-R5 treated and untreated cells at 5 nM, whereas compound UK-396794-27 achieved the same at 25 nM (Fig. 3.7E). Infection of untreated U87/CD4/CCR5 cells was completely inhibited by compound UK-403341-03 at 500 nM, whereas infection of ad-R5 treated cells was only reduced by 90%. Lower inhibitor concentrations (0.04, 0.2 and 1 nM) were also comparable, indicating that more CCR5 did not noticeably increase the concentration at which maximum inhibition occurred.
Figure 3.7. The efficiency of the small molecule inhibitors of CCR5 is dependent upon cell type.

Expression levels of CD4 (Q4120) and CCR5 (2D7) on NP2/CD4/CCR5 and U87/CD4/CCR5 cell lines were measured in comparison to the isotype control (IgG1), by flow cytometry (B). NP2/CD4 cells were pretreated with ad-CCR5 or media alone the day before infection with the R5 HIV-1 SF162, and titres compared to titre on stable NP2/CD4/CCR5 cells (C). Expression levels of CCR5 (horizontal axis) on U87/CD4/CCR5 cells untreated (left panel) or treated (right panel) with ad-CCR5 (vertical axis) (D). U87/CD4/CCR5 cells treated or untreated with ad-CCR5 were infected with the R5X4 HIV-1 2076,
3.2.8 Novel CCR5 inhibitors block infection of PBMCs by both R5 and R5X4 isolates of HIV-1

To gain a more realistic evaluation of this series of CCR5 inhibitors, their capacity to inhibit a range of R5 and R5X4 HIV-1 isolates on primary untransformed human PBMCs was tested. Previous reports have demonstrated that PBMC infection by R5 strains of HIV-1 is inhibited more efficiently by CCR5 inhibitors than cell line infection (Simmons et al., 1997). Indeed, PBMC infection by two R5 strains (SF162 and RU570) was more efficiently inhibited by RANTES, with approximately 90% inhibition being observed at 50 nM, in comparison to only 20% inhibition on the cell line U87/CD4/CCR5 at the same concentration (Figs. 3.5 and 3.8). Unlike inhibition observed with chemokines, inhibition of PBMC infection by the small molecule inhibitors of CCR5 was slightly less effective than that observed on cell lines, albeit still potent. Infection by SF162 was almost completely reduced by compounds UK-396794-27 and UK-400343-03 at 500 nM (Fig. 3.8A). On PBMCs, SF162 was relatively resistant to inhibition by compound UK-403341-03, with infection only being reduced by 30% at 500 nM, in comparison to 95% on cell lines (Fig. 3.8A). In contrast to R5 SF162, and RU570 infection of cell lines, infection of PBMCs by the R5 clade G isolate RU570 was relatively resistant to inhibition by all three small molecule inhibitors (Figs. 3.5A and 3.8A). All three compounds (UK-396794-27, UK-400343-03 and UK-403341-03) could only reduce infection between 40 and 50% at 500 nM.

PBMCs express both CCR5 and CXCR4, so inhibition of the R5X4 isolates 2076 and 2028 was inefficient, and reduced by no more than 60% (2076) or 25% (2028). However, in an excess of the CXCR4 inhibitor AMD3100, these isolates were potently inhibited. In comparison to R5 strains, inhibition of R5X4 isolates by RANTES and this series of CCR5 inhibitors was equally effective on both cell lines and PBMCs. In addition, PBMC infection by these R5X4 HIV-1 isolates was more sensitive to inhibition than R5 isolates, as seen on cell lines (Fig. 3.8B). Both 2076 and 2028 were completely inhibited by RANTES, UK-396794-27 and UK-400343-03 at 50 nM or less on cell lines and PBMCs (Figs. 3.4B and 3.8B). UK-403341-03, however, was extremely inefficient against these R5X4 strains on PBMCs, reducing infection by only 30% (2028) or not at all (2076). The order of efficiency of the compounds has thus remained consistent, irrespective of virus tropism and cell type.
Figure 3.8. Inhibition of PBMC infection by novel CCR5 inhibitors. PBMCs were pre-incubated for one hour with 0, 10, 100 or 1000 nM of UK-396794-27, UK-400343-03, UK-403341-03 or RANTES alone (A and B), or with AMD3100 at 1000 nM (C) before infecting with two R5 (SF162 and RU570; A) and two R5X4 (2076 and 2028; B and C) HIV-1 isolates. 100% infection for each virus represents RT activity in cell supernatant in the absence of inhibitors on day 9 of infection, was in the range of 500-2500 ng/ml, and was used to calculate all other values. All samples were done in triplicate, and error bars represent the standard error of the mean. Data is representative of at least two independent experiments.
3.2.9 Inhibition of macrophage infection by novel CCR5 inhibitors

Early studies demonstrated that macrophage infection via CCR5 was not sensitive to inhibition by β-chemokines (RANTES, MIP-1α, MIP-1β), although the chemokine analogue AOP-RANTES proved a highly potent inhibitor of M-tropic HIV-1 infection of macrophages (Simmons et al., 1997). The ability of small molecule antagonists of CCR5 to inhibit HIV-1 replication in primary macrophages was therefore investigated.

![Graph showing inhibition of macrophage infection by CCR5 inhibitors](image)

**Figure 3.9. Inhibition of macrophage infection by CCR5 inhibitors.** Macrophages were pre-incubated with UK-396794-27, UK-400343-03, UK-403341-03, RANTES or TAK-779 at 0, 10, 100 or 1000 nM before infecting with R5 (SF162 and RU570) or R5X4 (89.6 or P1019) isolates of HIV-1. Cells were stained for viral antigens on day 12 (89.6) or day 15 (SF162 and RU570) of infection. 100% infection represents the number of infected cells in the absence of inhibitor (80-200 FFU/ml). All samples were done in duplicate, and error bars represent the standard error of the mean of two independent experiments.
These compounds completely inhibited infection by the R5 isolate SF162, with UK-396794-27, UK-400343-03 and UK-403341-03 100% inhibition being observed for at 500 nM (Fig. 3.9A). In accordance with previous observations, the chemokine RANTES was not as effective as the small molecule inhibitors, achieving only 65% inhibition at 500 nM, whereas TAK-779 reduced infection of macrophages by 75% at the same concentration. The effect of the UK series of inhibitors on macrophage infection was more dramatic with the second R5 isolate, RU570. Infection was completely inhibited by both UK-396794-27 and UK-400343-03 at 5nM (Fig. 3.9A). Compound UK-403341-03 was less effective, only giving 80% inhibition at 500 nM. RANTES and TAK-779 gave similar inhibition profiles to that observed with SF162, reducing infection by 90% and 65%, respectively.

The R5X4 HIV-1 isolate 89.6, previously demonstrated to exploit both CCR5 and CXCR4 for infection of macrophages (Yi et al., 1998), and the pediatric R5X4 isolate P1019, were also efficiently inhibited by these compounds (Fig. 3.9B). UK-396794-27 and UK-400343-03 reduced infection of both isolates by approximately 95% at 500 nM. Compound UK-402343-03 was less efficient, reducing infection between 80% and 85% (89.6 and P1019) at 500 nM. Both RANTES and the CCR5 inhibitor TAK-779 were potent inhibitors of macrophage infection by the R5X4 strains, with almost 100% infection observed at 500 nM. Although CXCR4 is used by these strains for infection of macrophages, CXCR4-use by 89.6 is four-fold less efficient compared to CCR5-mediated infection (Yi et al., 1999). This high level of dependence upon CCR5 for macrophage infection explains the efficient inhibition seen with these CCR5 inhibitors. Moreover, the inability of these inhibitors to completely inhibit both P1019 and 89.6 infection of macrophages, when 89.6 inhibition was extremely potent on cell lines, can be explained by an inefficient exploitation of CXCR4.
3.3 Discussion

Small organic molecules that inhibit HIV entry would provide a significant advance on current anti-retroviral therapy that act on just two events in the HIV replication cycle – reverse transcription and virion maturation (section 1.5 and Fig. 1.7). Molecules falling into the category of HIV entry inhibitors include those able to bind to, and thus prevent infection via, one of the major HIV coreceptors CCR5, and include TAK-779, SCH-C (Baba et al., 1999; Strizki et al., 2001)(section 1.8.3.1). In addition to entry inhibitors targeting HIV coreceptors, peptides such as T20 block HIV infection by inhibiting the conformational changes in gp41 induced upon gp120/coreceptor interactions leading to fusion of viral and cellular membranes (section 1.8.3.2). Recently, a novel CCR5 inhibitor, UK-427857, was described by Pfizer Global Research and Development (Dorr et al., 2003). This prototype molecule is a highly potent small molecule inhibitor of CCR5, with broad cross-clade reactivity against HIV-1 and excellent oral bioavailability (Abel et al., 2003; Napier et al., 2003). In this chapter a novel series of heterocycle-substituted azabicycloalkane small molecule inhibitors of CCR5, of the same class as the current lead compound UK-427857, were characterised.

3.3.1 Specificity of small molecule inhibitors of CCR5

As the ultimate goal of chemokine receptor inhibitors is therapeutic use in addition to current antiretroviral therapy, it is important to determine their specificity with regards to binding other chemokine receptors. Both TAK-779 and SCH-C bind the chemokine receptor CCR2b, which has a high level of homology to CCR5 (approximately 76%), although only TAK-779 was reported to inhibit virus entry (SIV) via this coreceptor (Dragic et al., 2000; Rucker et al., 1996). In this study I tested one of the most efficient inhibitors of CCR5-mediated infection (UK-400343-03) for its coreceptor specificity. Compound UK-400343-03 had no inhibitory effect on cell lines expressing CCR3, CCR8, CXCR4, CXCR6, GPR1 or APJ, although it was able to inhibit CCR2b-dependent infection by the HIV-1 isolate GUN-1v. Infection of CCR2b expressing cells by GUN-1v was also inhibited by TAK-779, albeit less efficiently.

Although GUN-1v can exploit CCR5 for infection, it utilises CXCR4 much more efficiently, and in addition is able to exploit a range of alternative coreceptors (Shimizu et al., 1999). An increase in coreceptor use has been demonstrated to reduce affinity for CCR5 (Gorry et al., 2002a). As such, the broad coreceptor usage of this
isolate is likely to result in a comparatively weak interaction with each coreceptor (including CCR2b), and thus render it more sensitive to inhibition by weak, non-specific inhibitors like UK-400343-03. It has been demonstrated that TAK-779 binds within a small pocket formed by transmembrane helices 1, 2, 3, and 7 of CCR5 (Fig. 1.14)(Dragic et al., 2000). As CCR5 has a high level of homology to CCR2b (~76%), it is possible that the transmembrane helices of this receptor form a binding pocket similar enough to CCR5 to be blocked by both TAK-779 and UK-400343-03 (Rucker et al., 1996). Both TAK-779 and UK-400343-03 have similar molecular weights ($M_r$ 531.13 and $M_r$ 520.74) and contain a basic core. It is thus possible that this series of inhibitors bind to the small pocket occupied by TAK-779, and inhibit CCR5- and CCR2b-mediated HIV infection by a similar mechanism.

As chemokine receptors have crucial roles in the regulation of inflammatory responses, the specificity of a chemokine receptor inhibitor must be thoroughly assessed prior to use as a therapeutic tool. The cross-reactivity of UK-400343-03 with CCR2b could be a concern. However, the prototype compound of this series, UK-427857, does not inhibit binding of the chemokine MCP-3 to CCR2b and subsequent intracellular signalling events on CCR2b-expressing cells (P. Dorr, personal communication), suggesting that these compounds may specifically inhibit CCR2b-mediated infection without interfering with CCR2b function as a chemokine receptor. It has been demonstrated that the loss of a functional CCR5 has no detrimental effect upon the health of an individual. However, the lack of CCR5 in A32/A32 individuals may correlate with increased viral load in hepatitis C-positive individuals (Woitas et al., 2002). Considering the high rate of co-infection with HIV and hepatitis C, this could be a concern (Ockenga et al., 1997; Rockstroh, Woitas, and Spengler, 1998; Stary et al., 1992). Moreover, it is possible that the lack of CCR5 in A32/A32 individuals is compensated by unknown mechanisms during development of the immune system. Therefore, CCR5+ individuals may not carry the capacity to compensate for loss of CCR5 function, and unexpected complications may result in the use of CCR5 inhibitors in vivo.

### 3.3.2 Inhibition of diverse HIV and SIV isolates by novel CCR5 inhibitors

The effect of the previously described small molecule inhibitors of CCR5 (TAK-779 and SCH-C) on R5-using HIV-1 isolates has been extensive; however, their effect
on R5X4 isolates has not been reported (Baba et al., 1999; Dragic et al., 2000; Strizki et al., 2001; Trkola et al., 2002). It is known however, that CCR5 inhibitors such as the β-chemokine RANTES, inhibit R5X4 infection of CCR5+ cell lines more effectively than R5 viruses (Kledal et al., 1997). As a result, the effect of this series of inhibitors on a diverse range of HIV and SIV isolates, of varying coreceptor usage requirements, was determined. All three compounds investigated here (UK-396794-27, UK-400343-03 and UK-403343-03) were consistently more effective against a range of R5X4 HIV-1 isolates than a range of R5 isolates, a trend that was also observed with HIV-2 isolates. When HIV evolves to exploit CXCR4 in addition to CCR5 for entry, it is likely that the affinity for CCR5, and thus the strength of envelope interaction with CCR5, is compromised. As previously demonstrated, this results in isolates using more than one coreceptor for entry being more susceptible to inhibition by CCR5-specific inhibitors than isolates exploiting CCR5 alone (Gorry et al., 2002a).

A concern with the use of coreceptor-specific HIV inhibitors in a therapeutic setting is that the constant presence of a potent CCR5 inhibitor could force the evolution of viral escape mutants. Such mutants could escape inhibition by adapting to exploit CXCR4, and could potentially induce a faster progression to AIDS (Bjorndal et al., 1997). Such escape has been previously demonstrated in a hu-PBL-SCID mouse model with the potent CCR5 antagonist AOP-RANTES, and another RANTES derivative N\(^\text{\(\alpha\)}\)-nonanoyl-RANTES[2-68] (NNY-RANTES)(Mosier et al., 1999). This study investigated the effect of these two inhibitors on the infection of SCID-huPBL mice by the R5 molecular clone 242, an isolate requiring very few amino acid substitutions to acquire CXCR4-use. Only 4 out of 10 mice treated with NNY-RANTES became infected with clone 242, and only 2 of these carried V3 sequence changes that conferred a switch to R5X4. The virus variant with a switched genotype did rapidly revert back to wild-type upon in vitro culture in the absence of inhibitor, implying that coreceptor switch under treatment with a CCR5 inhibitor may induce evolution of less ‘fit’ escape variants.

A second mechanism for viral escape from a coreceptor inhibitor is the evolution to use CCR5 despite the presence of a CCR5-specific inhibitor, as reported for AD101, a derivative of the potent CCR5 antagonist SCH-C (Trkola et al., 2002). An uncloned primary R5 HIV-1 isolate, CC1/85, was passaged once a week in PBMCs in the
presence or absence of AD101 at a concentration inhibiting >90% infection. Following 19 passages, virus grown in the presence of AD101 was replicating to levels seen in the absence of inhibitor, and was deemed resistant. Subsequent analysis of this isolate found it to be resistant to inhibition by the original CCR5 inhibitor SCH-C, yet sensitive to inhibition by RANTES and the CCR5 mabs 2D7 and PA14, and unable to replicate in CXCR4-expressing Δ32/Δ32 CCR5 PBMCs. Such evolution of HIV-1 to use CCR5 in the presence of a CCR5 inhibitor could explain the infection of the 2 SCID-huPBL mice in the NNY-RANTES study described above that became infected in the presence of the inhibitor, yet showed no evidence of a coreceptor switch (Mosier et al., 1999).

Overall, the literature suggests that evolution of HIV-1 to undergo a coreceptor switch from R5 to R5X4 or X4 may not necessarily be a rapid consequence in patients being treated with coreceptor-specific inhibitors. Evolution of HIV to exploit alternative regions of CCR5, and the corresponding ability of HIV-1 to exploit low cell surface concentrations of CCR5 (Trkola et al., 2002), is an area that requires more investigation (Farber and Berger, 2002). The series of inhibitors described in this chapter has been demonstrated to inhibit infection by a very diverse range of HIV-1, -2 and SIV strains. This range includes the isolate SIVmac239, which preferentially utilises the second extracellular domain of CCR5 (CCR5 E2) for infection (Edinger et al., 1997b), and may indicate that viral escape by utilisation of different regions of CCR5 is not a simple mechanism.

3.3.3 Effect of CCR5 expression levels on inhibitor efficiency

The potency of these CCR5 inhibitors was influenced by the target cell of infection. The cell line U87/CD4/CCR5 was consistently more sensitive to inhibition by these heterocycle substituted azabicycloalkane inhibitors than the similar astrogliaoma-derived NP2/CD4/CCR5 cell line. Flow cytometric analysis of these cell lines showed similar expression levels of CD4, but significantly higher levels of CCR5 on the NP2 cell line. Increasing cell surface expression of CCR5 on the U87 cell line with an adenovirus-CCR5 vector did not render these cells less sensitive to the CCR5 inhibitors, indicating the possible existence of intracellular factors influencing sensitivity to CCR5 inhibitors. Regardless, the series of inhibitors tested above carry the capacity to block infection of cells with high cell surface CCR5 expression levels. However, it cannot be excluded that some cell populations in vivo may be more resistant to blockade by the same inhibitors.
3.3.4 Effect of small molecule inhibitors of CCR5 on primary cell infection

It has been demonstrated that PBMC infection by R5 isolates of HIV-1 is more sensitive to inhibition by β-chemokines, such as RANTES and MIP-1α/β, than cell line infection (Simmons et al., 1997). Although PBMC infection was slightly less sensitive to inhibition than cell lines, this series of inhibitors was still very potent against both R5 and R5X4 isolates of HIV-1. These data agrees with that reported by Strizki et al, who showed similar IC50 figures for inhibition of single-cycle replication of HIV pseudotypes in U87/CD4/CCR5 cells, and replication of a range of diverse HIV isolates in PBMCs (Strizki et al., 2001). An intriguing exception to the efficiency of these small molecule inhibitors against PBMC infection was observed with the clade G HIV-1 isolate RU570 on PBMCs. This isolate was very sensitive to inhibition by RANTES, yet was remarkably insensitive to inhibition with all three small molecule inhibitors, despite being well inhibited on cell lines. The reason behind this remains unclear, although it has been reported to be the only HIV-1 isolate of a large and diverse range of strains to be insensitive to SCH-C (Strizki et al., 2001). As seen with inhibition of cell line infection, the order of efficiency of the three CCR5 inhibitors was consistent. PBMC infection by the clade G isolate RU570 was the least sensitive to inhibition by UK-397296-27, UK-400343-03 and UK-403341-03, although RANTES did completely inhibit this virus at high concentrations.

The CCR5 ligands RANTES, MIP-1α and MIP-1β have been shown to be less effective at suppressing CCR5-mediated infection of primary macrophages in comparison to PBMCs (Dragic et al., 1996; Ketas et al., 2003; Moriuchi et al., 1996; Simmons et al., 1997). These observations highlight the possibility that the therapeutic use of anti-CCR5 drugs may reduce HIV replication in some cell types (PBMCs), but leave a large population of cells still vulnerable to infection (macrophages). This has implications when the potential of macrophages as reservoirs for HIV in patients on HAART is considered (Blankson, Persaud, and Siliciano, 2002). I confirmed these observations in this study, which demonstrates that infection of macrophages by both SF162 and RU570 (R5 isolates) is less sensitive to inhibition by RANTES. In contrast to RANTES and SCH-C, both of which are more effective inhibitors of PBMC than macrophage infection all compounds in this series of inhibitors were more effective against RU570 infection of macrophages than PBMCs (Ketas et al., 2003). SF162 infection of these two cell types was equally well inhibited. It is not clear why
differences were observed between the two R5 strains; however, this study and others have demonstrated RU570 to be somewhat unusual with regards to inhibition by CCR5 inhibitors (Strizki et al., 2001). The inhibitor TAK-779 did inhibit these R5 isolates on macrophages, although the inhibition profile was consistently less effective than the CCR5 small molecules, and more closely resembled inhibition seen with RANTES.

Like PBMCs, macrophages express both CCR5 and CXCR4; however, the ability of T-tropic and/or dual-tropic isolates to infect macrophages via CXCR4 is variable (Schuitemaker et al., 1991; Simmons et al., 1998; Yi et al., 1998). Although a range of R5X4 isolates have been shown to routinely infect macrophages, many of the isolates tried within this study were unable to replicate to high enough levels in macrophages to gain accurate inhibition data, although efficiency of infection was increased upon spinoculation (O'Doherty, Swiggard, and Malim, 2000). For example, 2076 and 2028 gave very low levels of infection, despite relatively high titres on coreceptor expressing indicator cell lines and previous evidence for macrophage-tropism (Simmons et al., 1998). The lack of macrophage infection by GUN-1v correlates with a single change in the V3 loop, which also confers infectivity for various brain cell cultures (Chapter 4)(McKnight et al., 1995). The primary isolate HAN-2 also infects the same range of brain cell types as GUN-1v, and thus tropism characteristics may preclude macrophage replication. This issue relates to coreceptor use, and will be discussed in Chapter 4.

The R5X4 isolate 89.6 has previously been demonstrated to efficiently infect macrophages via both CCR5 and CXCR4, although CXCR4-mediated infection was shown to be up to four-fold less efficient than CCR5-mediated infection (Collman et al., 1992; Yi et al., 1999; Yi et al., 1998). Indeed, efficient infection of macrophages by 89.6, as well as the paediatric isolate P1019, was observed. This infection was, surprisingly efficiently inhibited by all CCR5 small molecules, including TAK-779, and also by RANTES. Relatively inefficient use of CXCR4 by these isolates would explain the residual low level infection of macrophages observed in the presence of CCR5 inhibitors.
3.4 Summary

In conclusion, the small molecules UK-396794-27, UK-400343-03 and UK-403341-03 form a series of potent CCR5 inhibitors that are extremely effective at inhibiting infection by a very diverse set of HIV and SIV isolates. Inhibition of infection was demonstrated for a range of cell lines and primary cell cultures, including T-cells and macrophages that represent the main cell types targeted and infected by HIV \textit{in vivo}. 
CHAPTER 4

Identification of a subset of HIV and SIV strains able to exploit an alternative coreceptor on primary human brain and lymphoid cells

4.1 Introduction

CCR5 is the major coreceptor used during HIV transmission and CCR5-using strains remain present throughout infection (Schuitemaker et al., 1991). HIV variants able to exploit CXCR4 emerge late in disease in up to 50% of AIDS patients, and this coreceptor switch correlates with disease progression (Connor et al., 1997; Scarlatti et al., 1997; Tersmette et al., 1988). However, since a coreceptor switch from R5 to R5X4 or X4 strains is not observed in all HIV+ patients, this cell tropism change is not the sole determinant for progression to AIDS (de Roda Husman et al., 1999). A change in coreceptor use is thought to confer a broadening of tropism in the late stages of disease, enabling the infection of new target cell populations. For example, strains able to exploit CXCR4 in vivo are proposed to target largely uninfected CXCR4+CCR5 T-cell populations e.g. the naïve T-cell population (Blaak et al., 2000; Ostrowski et al., 1999).

Although CCR5 and CXCR4 are the major coreceptors used in vivo there are at least twelve other members of the chemokine receptor family, and related ‘orphan’ receptors, that can support infection of CD4+ indicator cell lines in vitro (section 1.6.2.3). In general, HIV-2 and SIV strains use a wider range of these alternative coreceptors than HIV-1 in vitro, frequently as efficiently as they use CCR5 and/or CXCR4 (Clapham and McKnight, 2002; Reeves and Doms, 2002). Such alternative coreceptors appear to be rarely used during in vivo replication; however, there is as yet no convincing evidence to indicate that these receptors are of no consequence in vivo. Thus, the ability of HIV to use these other coreceptors on indicator cell lines does not seem to provide a true indication of coreceptor usage properties in vivo.

For HIV-1, there is some evidence that alternative coreceptors (other than CCR5 and CXCR4) are used in vivo (Lee et al., 2000; Sharron et al., 2000; Zhang, Y. et al., 1998b), but the contribution of such alternative coreceptors to in vivo viral replication is currently thought to be insignificant. Nevertheless, the evolution of viral quasispecies to exploit other coreceptors could further increase the target cell population including
possible reservoir cells in immunoprivileged sites (Blaak et al., 2000; Brack-Werner, 1999; Ostrowski et al., 1999). For example, astrocytes, particularly in paediatric cases, become infected to a low level during the later stages of disease (Sabri et al., 1999; Tomatore et al., 1994a; Wiley et al., 1986). In addition, HIV-1 proteins and nucleic acids have been detected within the testes of infected men (da Silva et al., 1990; Muciaccia et al., 1998), and HIV-2/SIV have been shown to infect Leydig cells in vitro despite no detectable expression of common HIV coreceptors such as CCR5 and CXCR4 (Willey et al., 2003b).

This chapter describes the analysis of a range of untransformed human cell cultures for the expression of alternative coreceptors able to support HIV and SIV infection. Astrocytes, established either from adult temporal lobectomy samples or foetal tissue, untransformed brain microvascular endothelial cells (BMVECs), and Leydig cells are negative for one or both of the major coreceptors CCR5 and CXCR4, and are thus suitable target cells to test for the presence of alternative coreceptors. A subset of HIV and SIV strains were able to exploit an unknown coreceptor on these cells, and several of these were found to replicate in CCR5+ PBMCs where infection via CXCR4 is blocked by the bicyclam AMD3100. The capacity of HIV to exploit such naturally expressed coreceptors may provide a stronger indication for their use in vivo.
4.2 Results

4.2.1 Human Leydig cells can be productively infected by HIV-2 and SIV strains

Some HIV-infected men suffer from testicular dysfunction, and HIV-1 proteins and nucleic acids have been detected within the testes (da Silva et al., 1990; Dejucq and Jegou, 2001; Muciaccia et al., 1998). Primary human Leydig cells were therefore tested for their ability to support infection by HIV and SIV. As Leydig cells do not express CD4, and most HIV-1 strains identified to date are dependent upon CD4 for infection, an adenovirus-CD4 vector (Ad-CD4) was used to express CD4 and analyse the expression of functional HIV/SIV coreceptors (Habasque et al., 2002; Reeves et al., 1999).

Leydig cells were isolated from normal testes tissue removed from prostate cancer patients (section 2.3.5.5) and exposed to a range of HIV-1, HIV-2 and SIV strains with well-defined coreceptor usage patterns (Table 2.7). As seen in Fig. 4.1A, infection of the cell line NP2/CCR5 with Ad-CD4 was sufficient to confer infection by the R5 HIV-1 strain SF162 to levels comparable to that seen on NP2/CD4/CCR5 cells. None of the HIV-1 strains tested, irrespective of cell tropism (SF162, R5; 2044, X4; GUN-1WT, R5X4), were able to infect Leydig cells either with or without CD4, despite the contamination of Leydig cell preparations with low levels (<1%) of macrophages (Willey et al., 2003b)(Fig. 4.1B). The HIV-2 strains ROD B and JAU, both of which are CD4-independent, infected Leydig cells in the presence and absence of CD4 (Fig. 4.1C). The HIV-2 strain TER and SIV strains Man4 and 17E-fr were only able to infect upon expression of CD4; however, the SIV strain 17E-cl infected equally well in the presence or absence of CD4 (Fig. 4.1B).

To identify the coreceptor(s) being used for infection, a range of chemokine receptor ligands were tested for their capacity to block infection by HIV-2 ROD B. Infection by ROD B occurs in the absence of CD4 so cells were not pre-treated with Ad-CD4. Infection was unaffected by the chemokines RANTES (binds CCR5, CCR1, CCR3 and CCR9) and eotaxin (CCR3, D6)(Fig. 4.1D). However, the CXCR4 antagonist AMD3100, and the CD4 mab Q4120 (binds to D1 of CD4) reduced infection to approximately 20% of levels seen in the absence of inhibitor, in agreement with previously demonstrated RT-PCR results (Willey et al., 2003b).
Figure 4.1. **Leydig cells are susceptible to infection by HIV-2 and SIV isolates.** The activity of Ad-CD4 was tested by pre-treating NP2/CCR5 cells with Ad-CD4 for three hours, washing and incubating overnight. Ad-CD4 pre-treated and untreated NP2/CCR5 cells, as well as NP2 cells stably expressing CD4 and CCR5 were exposed to the R5-using HIV-1 SF162 for 3 hours, and infected cells detected by p24 immunostaining after 72 hours (A). Leydig cells were exposed to a range of HIV-1, HIV-2 and SIV strains with titres between $1 \times 10^3$ and $1 \times 10^5$, either with or without pre-treatment with Ad-CD4 (B), or infected with the HIV-2 ROD B for 3 hours without Ad-CD4 pre-treatment (C). Infected cells were detected by p24 immunostaining and titres given in FFU/ml. Sensitivity of Leydig cell infection to a range of inhibitors was determined by pre-treating for 1 hour with chemokines (100 nM), coreceptor ligands (1000 nM) or antibodies (20\(\mu\)g/ml) before exposing to HIV-2 ROD B (D). 100% infection represents RT activity in culture supernatant at day 7 of infection in the absence of inhibitors, was in the range of 20-90 pg/ml, and was used to calculate all other values. A titre of 0 indicates <10 FFU/ml.
It is possible that ROD B infection is partially attributed to infection of contaminating macrophages, explaining the observed inhibition with Q4120. However, if contaminating macrophages were entirely responsible for the observed Leydig cell infection, other isolates able to exploit CCR5 (SF162, GUN-1WT) or CXCR4 (2044) should productively infect the Leydig cell cultures, even in the absence of adenovirus-mediated CD4 expression. This was not observed. In addition, infection was not completely reduced by the CXCR4-specific inhibitor AMD3100 (Hatse et al., 2002), implying that an alternative coreceptor contributes to ROD B Leydig cell infection.

4.2.2 BMVECs support infection by a subset of HIV-1, HIV-2 and SIV isolates

HIV-1 is found within both the cerebral spinal fluid (CSF) and cells of the central nervous system (CNS)(section 1.2.1.4). As brain microvascular endothelial cells (BMVECs) are one of the major cell types found in the blood:brain barrier (BBB), their susceptibility to a panel of HIV-1, -2 and SIV isolates was tested. As seen with infection of Leydig cells, HIV-1 isolates able to exploit either CCR5 (SF162) or CXCR4 (2044) alone were unable to infect untransformed BMVECs (Fig. 4.2A). In addition, HIV-2 isolates that predominantly use CCR5 or CXCR4 (ALI, SAB, MIL) were also unable to infect these cells (Fig. 4.2A). In contrast, efficient replication was seen in BMVECs by two R5X4 HIV-1 isolates (GUN-lv and HAN-2), three HIV-2 isolates exploiting CCR5 (TER) or CCR5 and CXCR4 (JAU and MLC) as well as two broadly tropic SIV isolates (SIVmac316 and SIV17E-fr)(Fig. 4.2A). Although all but one isolate that infected BMVECs were R5X4, other R5X4 isolates (HIV-1 89.6 and HIV-2 ETP) were unable to infect BMVECs. All strains found to successfully infect these cells efficiently exploit CCR8 and/or GPR1 for infection of indicator cell lines, whereas 89.6 and ETP do not (Table 2.7).

As with Leydig cell infection, the identity of the alternative coreceptor(s) being exploited for infection of BMVECs was investigated by determining sensitivity to inhibition by coreceptor ligands. Because all isolates able to infect BMVECs were able to exploit CCR3, CCR8 and GPR1 for infection (in addition to CCR5 and sometimes CXCR4), ligands binding these receptors were included in the inhibition assays. The activity of all coreceptor-specific chemokines was determined by carrying out control inhibitions of coreceptor expressing cell lines. CCR3- and CCR8-mediated HIV infection was selectively inhibited by the ligands eotaxin and I309, respectively (Fig. 4.2B).
Figure 4.2. BMVECs support infection by a subset of HIV-1, HIV-2 and SIV isolates. BMVECs were exposed to HIV and SIV strains and infected cells were detected by immunostaining after 72 hours (A). Sensitivity of BMVEC infection to chemokine inhibition was determined by pre-incubating cells with coreceptor ligands (200 nM) or CD4 mabs (10 µg/ml) before exposing to HIV-1 GUN-lv (B). Infections were performed in duplicate and error bars represent the standard error of the mean. A titre of 0 indicates <10 FFU/ml was observed. Results are representative of two independent experiments.
Figure 4.2C. BMVEC infection is inhibited by vMIP-I. Sensitivity of BMVEC infection to chemokine inhibition was determined by pre-incubating cells with coreceptor ligands (200 nM) or CD4 mabs (10 μg/ml) before exposing to HIV-1 GUN-Lv (C). Infections were performed in duplicate and error bars represent the standard error of the mean. A titre of 0 indicates <10 FFU/ml was observed. Results are representative of two independent experiments.
In comparison, vMIP-I (HHV8-encoded chemokine) inhibited infection of control cell lines expressing CCR8, GPR1 and CXCR6 as previously reported (Simmons et al., 2000), and weakly blocked CCR3-mediated infection (Fig. 4.2B). On BMVECs, the HIV-1 strain GUN-1v was completely inhibited by vMIP-I (HHV8-encoded chemokine), but unaffected by the ligands eotaxin (CCR3), AOP-RANTES (CCR5), AMD3100 (CXCR4) and I309 (CCR8) (Fig. 4.2C). A second HHV8-encoded chemokine analogue vMIP-II, which inhibits infection via CCR3, CCR5 and CXCR4, had no effect on infection, confirming the results seen with the more specific chemokine receptor ligands. The CD4 mabs Q4120 (binding domain D1 on CD4) and 5A8 (binding domains D2 and D3) reduced BMVEC by more than 95%, indicating that CD4 was present on the cell surface at levels high enough to support the efficient infection observed by HIV/SIV strains not previously shown to be CD4-independent (Fig. 4.2A). The corresponding isotype control antibody, IgG1, had no effect on BMVEC infection. Complete inhibition of BMVEC infection by vMIP-I, as well as the lack of inhibition by potent ligands for CCR5 and CXCR4, confirms that an alternative coreceptor is responsible for virus entry.

4.2.3 Cultured astrocytes do not express CD4 or CCR5

Astrocytes do not usually express CD4 and therefore infection by HIV-1 is inefficient (Sabri et al., 1999; Tornatore et al., 1994a). The chemokine receptor-expression profile of astrocytes is controversial, and the role of coreceptors in the low-level infection is unclear (section 1.2.1.4) (Berger, Murphy, and Farber, 1999; Sabri et al., 1999). The cells cultured from foetal tissue (sample F01) and adult temporal lobectomies (samples 001A and 004.2) were confirmed to be astrocytes by testing for expression of glial acidic fibrillary protein (GFAP), an astrocyte-specific cytoskeletal protein. Foetal astrocytes were strongly positive, and a well-defined cytoskeletal structure was observed (Fig. 4.3A). In contrast, both adult cultures 001A and 004.2 showed weaker and more diffuse staining (Fig. 4.3B and C, respectively), consistent with the observations of Marriott et al who describe a differential GFAP morphology in astrocytes dependent upon the astrocyte precursor cell and the age of the tissue (Marriott, Hirst, and Ljungberg, 2000). The control cell line NP2/CD4/CCR5 was negative for GFAP (Fig. 4.3D). The chemokine receptor expression profile of human astrocytes is controversial, so the expression of CCR5, CXCR4 and CD4 on each of the untransformed astrocyte cultures was tested immunohistochemically. The purified foetal astrocytes F01 expressed neither CCR5 nor CD4, but were weakly positive for
CXCR4 as shown previously (Fig. 4.3E) (Reeves et al., 1999), in comparison to a control cell line expressing high levels of CXCR4 (NP2/CD4/CXCR4) (Fig. 4.3H). CD4, CCR5 and CXCR4 were not detected on either adult brain-derived cultures 001A or 004.2 (4.3F and G), but were present on control cell lines (Fig. 4.3H).

Figure 4.3. Untransformed astrocytes do not express CD4 or CCR5. Foetal astrocytes (A), adult astrocyte cultures 001A (B) and 004.2 (C), and control NP2/CD4/CCR5 cells (D) were stained for GFAP, a marker for astrocytes. The presence of the HIV receptors CD4 (mab Q4120), CCR5 (mab 2D7) and CXCR4 (mab 12G5) was determined on foetal astrocytes F01 (E), adult astrocytes 001A (F) and 004.2 (G), and control cells NP2/CD4 expressing CCR5 (H; top and middle panel) or CXCR4 (H; bottom panel). Nuclei of antigen-negative cells were stained with propidium iodide (red).
4.2.4 CD4 expression on transformed astrocyte cultures confers sensitivity to the same subset of HIV and SIV isolates able to infect BMVECs

Cell surface expression of a chemokine receptor is not sufficient to demonstrate its use as an HIV coreceptor (Dittmar et al., 1997). Indeed, studies have demonstrated that embryonic astrocytes are not susceptible to infection by either M- or T-tropic isolates of HIV-1 despite expression of functional coreceptors (Boutet et al., 2001).

Figure 4.4. CD4 expression on astrocytes confers susceptibility to infection with HIV/SIV. GFAP⁺ adult astrocytes 001A were pre-treated with Ad-CD4, infected with HIV-1, and immunostained for intracellular p24 after 72 hours (A). The titre of each HIV/SIV isolate (FFU/ml) was assessed on adult astrocytes 001A and 004.2 (B) and foetal astrocytes F01 (C) pre-treated with Ad-CD4. All HIV/SIV isolates had titres between $10^5$ and $5 \times 10^5$ FFU/ml on NP2/CD4 coreceptor-expressing indicator cells (Table 2.7). A titre of 0 indicates <10 FFU/ml. Data are representative of three independent experiments.
Low-level infection of astrocytes is observed during the early stages of disease in HIV-1 infected individuals (Tornatore et al., 1994b) despite the fact that astrocytes do not widely express CD4 (Ma, Geiger, and Nath, 1994), and that the majority of HIV-1 isolates are highly CD4-dependent. As with CD4-negative Leydig cell cultures, untransformed foetal and adult GFAP+ astrocyte cultures were pre-infected with Ad-CD4. CD4-expressing astrocytes were then used to screen a range of HIV and SIV isolates for their capacity to use naturally expressed alternative coreceptors. Adult astrocyte cultures (001A and 004.2) were resistant to HIV-1 strains that strictly use CCR5 (SF162) or CXCR4 (2044) (Fig. 4.4A). As observed with BMVEC infection, both adult astrocyte cultures were sensitive to infection by several HIV/SIV strains. Three R5X4 HIV-1 strains (P1019, HAN-2 and GUN-1v) and several HIV-2 and SIV strains (TER, ETP, SIVman4 and SIVmac17E-fr) were able to exploit astrocyte-expressed coreceptors, and efficiently infected adult astrocytes 001A and, to a lesser extent, 004.2 (Fig. 4.4A and B). As the foetal astrocyte culture F01 weakly expressed CXCR4 (Fig. 4.3E), adenovirus-mediated expression of CD4 resulted in infection by all isolates able to use CXCR4 including the HIV-1 X4 isolate 2044 (Fig. 4.4C). However, since TER does not use CXCR4 on standard CD4+ indicator cell lines (Reeves et al., 1999), and CXCR4 use by SIVman4 is inefficient and at least 1000 times less than CCR5 use, it is likely that the observed infection of foetal astrocytes by these strains is due to the exploitation of an alternative coreceptor.

4.2.5 Infection of foetal and adult astrocytes by HIV/SIV strains in the absence of CD4 expression

Low-level CD4-independent infection of brain cultures has been reported for various HIV and SIV strains (Clapham et al., 1996; Sabri et al., 1999). Therefore, foetal and adult astrocytes were tested for their capacity to support infection by the same panel of HIV and SIV isolates in the absence of adenovirus-mediated CD4 expression. These cultures were negative for CD4 expression by immunohistochemical methods, and infection by most strains was weak, as expected (Fig. 4.5). The R5 HIV-1 SF162 and X4 2044, both of which are dependent on CD4 for infection, were unable to infect the adult astrocytes, as was the R5X4 HIV-2 strain ETP (Fig.4.5A). The R5X4 HIV-1 isolates HAN-2 and P1019, as well as the HIV-2 strain TER and the SIV isolates Man4 and 17E-fr, were variable in their ability to infect the two adult astrocyte cultures. The HIV-1 strain HAN-2 was able to infect the adult astrocyte culture 001A to a low level, yet unable to infect culture 004.2. In contrast, the isolate P1019 infected 004.2, not
001A (Fig. 4.5A). The HIV-1 isolate GUN-1v infected both adult astrocyte cultures 004.2 and 001A to high levels, even though it has not been previously demonstrated to infect CD4-independently (Liu et al., 2000a). SIVman4 infected the adult astrocyte culture 001A more efficiently than 004.2, whereas SIV17E-fr only infected 001A (Fig. 4.5A).

**Figure 4.5. Susceptibility of astrocytes to HIV/SIV in the absence of CD4 expression.** Adult astrocytes 004.2 and 001A (A), and foetal astrocytes F01 (B) were exposed to a panel of HIV and SIV isolates for three hours. Infected cells were detected by immunostaining for intracellular viral p24 after 72 hours. Titres are given in FFU/ml, and are representative of three independent experiments. A value of 0 indicates a titre of <10 FFU/ml. All viruses tested had infectivity titres between $10^4$ and $5 \times 10^5$ FFU/ml on NP2/CD4 coreceptor-expressing indicator cells.

The reason for this variability in infectivity between the two adult astrocyte cultures is unclear. The two astrocyte cultures may express different levels of the
putative active coreceptor(s), or it may be expressed as different isoforms variable in their capacity to support HIV/SIV. Another possibility is that the cells may express different levels of unknown cellular factors that may influence coreceptor activity or a post-entry restriction to infection by HIV/SIV.

The foetal astrocyte culture FOI was resistant to infection by HIV-1 SF162 (R5) and 2044 (X4), as well as the R5X4 isolate P1019, but was able to support inefficient infection by the HIV-1 isolates GUN-1v and HAN-2 (Fig. 4.5B). The strain HAN-2 has previously been reported to infect CD4+ cells via CXCR4, but only with sCD4 pre-treatment (Reeves et al., 1999). Although these foetal astrocytes express low levels of CXCR4, CD4 was not detected (Fig. 4.3E). Neither SIVman4 nor SIV 17E-fr were able to infect this culture, despite the fact that they are able to exploit CXCR4 (albeit weakly) and able to infect cells in a CD4-independent manner (Edinger et al., 1997b; Reeves et al., 1999).

4.2.6 Infection of foetal and adult astrocytes is sensitive to inhibition by chemokine receptor ligands

To identify the possible coreceptor(s) being exploited for infection of foetal and adult astrocytes, a range of chemokine receptor ligands were tested for their capacity to block infection by the HIV-1 strain GUN-1v. When adult astrocytes 001A were pre-treated with Ad-CD4, GUN-1v infection was reduced to approximately 50% of levels seen in the absence of inhibitors by both vMIP-I (binds CCR8, GPR1 and CXCR6) and RANTES (binds CCR5, CCR1, CCR3 and CCR9). The ligand eotaxin (CCR3, D6) also reduced infectivity to approximately 60% (Fig. 4.6A).

The CCR5 small molecule inhibitor TAK-779 was unable to inhibit astrocyte infection, despite being able to block infection of CCR5-expressing cell lines (Fig. 4.2B). This, in conjunction with the observation that the CCR5 mab 2D7 was ineffective against astrocyte infection in comparison to the IgG1 isotype control, confirms that inhibition observed by RANTES is not due to the expression and use of CCR5 for infection. 001A infection was unaffected by the CXCR4 antagonist AMD3100 and the CCR8-specific ligand I309. Infection of GFAP+ astrocytes therefore identifies the same subset of HIV-1, HIV-2 and SIV strains that infect BMVECs.

The same set of chemokines that weakly inhibited infection of CD4+ adult
astrocytes 001A (vMIP-I, eotaxin and RANTES) also reduced infection in the absence of CD4 (Fig. 4.6B). RANTES reduced infection to approximately 40% of control levels in the absence of inhibitor, as did eotaxin and vMIP-I. I309 lowered infection by approximately 35%.

Figure 4.6. Astrocyte infection is sensitive to inhibition by chemokines. Adult astrocytes 001A pre-treated (A) or untreated (B) with Ad-CD4, and foetal astrocytes F01 (C) were pre-incubated with a range of coreceptor ligands or small-molecule inhibitors before infecting with HIV-1 GUN-lv. Infected cells were detected by immunostaining for viral antigens 72 hours post-infection. 100% infection represents titre in the absence of inhibitor, and was approximately 150 FFU. All samples were done in duplicate, and the error bars represent the standard error of the mean.
Interestingly, infection of CD4⁺ 001A cells was completely blocked by the CD4 mab Q4120 (Fig. 4.6B) indicating that these astrocytes express low levels of CD4. These levels are undetectable by immunostaining (Fig. 4.3F), yet sufficient enough to explain the low-level infection observed by the CXD4-dependent HIV-1 GUN-1v in the absence of adenovirus-mediated CD4 expression. As observed with the CD4⁺ astrocytes, infection was unaffected by the CCR5 mab 2D7 in comparison to the isotype control. Infection of the foetal astrocyte preparation F01 by GUN-1v was reduced to less than 10% of control levels by AMD3100 (Fig. 4.6C). The addition of vMIP-I to AMD3100 gave no further inhibition of F01 infection. Moreover, infection of foetal astrocytes was only minimally affected by the chemokines RANTES, I309 and vMIP-I, confirming that CXCR4 conferred the majority of infection in these cells.

4.2.7 A subset of HIV and SIV isolates can infect Δ32/Δ32 CCR5 PBMCs with CXCR4 blocked by AMD3100

Experiments described above provide evidence that BMVECs, astrocytes and Leydig cells naturally express alternative coreceptors that are functional for a subset of HIV-1, HIV-2 and SIV strains. There is some evidence for infection of both astrocytes and Leydig cells in vivo, and although infection of these cells may have physiological effects (such as testosterone imbalances) they are not major cellular targets for HIV-1 in vivo (section 1.2.1). To determine the use of alternative coreceptors on a more physiologically relevant cell type, the ability of this HIV and SIV subgroup to exploit an alternative coreceptor(s) on PBMCs was examined. For this, PBMCs prepared from an individual homozygous for the 32-base pair deletion in CCR5 (Δ32/Δ32 CCR5) were challenged in the presence of AMD3100 to block CXCR4.

GUN-1v is a T-cell line adapted virus originally isolated from GUN-1WT by its ability to replicate in brain tumour cell lines (Takeuchi et al., 1991). GUN-1v possesses only a single amino acid difference between GUN-1WT within the envelopes V3 loop, and this adaptation confers a much broader coreceptor usage profile on GUN-1v, although the ability to replicate in primary macrophages is lost (McKnight et al., 1995). Although GUN-1v replicates in PBMCs, it undergoes a lag phase before replication ensues, likely representing adaptive processes as observed in the initial isolation of GUN-1v from GUN-1WT. As the coreceptor usage properties of the adapted GUN-1v virus are not characterized at this point, data of GUN-1v infection of Δ32/Δ32 CCR5 PBMCs is not presented here.
Figure 4.7. Infection of Δ32/Δ32 CCR5 PBMCs by HIV and SIV strains with CXCR4 blocked.

Δ32/Δ32 CCR5 were pre-incubated for 1 hour with media alone or the inhibitor AMD3100, vMIP-I or AMD3100 + vMIP-I at 1000 nM before infecting in triplicate with HIV-1 (2044, HAN-2 or P1019), HIV-2 (TER or ETP) or SIV (man4). All virus stocks had titres between 10^4 and 10^5 FFU/ml on NP2/CD4/CCR5 or NP2/CD4/CXCR4 indicator cell lines. Supernatant was removed every three days up to day 18, and virus production was measured by RT ELISA. All points represent the average of triplicate samples, and results are representative of at least two independent infections.

On Δ32/Δ32 CCR5 PBMCs, HIV-1 2044 (X4) was efficiently inhibited by AMD3100, but not by vMIP-I, although it did delay the rate of replication of this isolate (Fig. 4.7). Replication of the HIV-1 strain HAN-2, and the HIV-2 strain TER was reduced, but not completely inhibited, by both AMD3100 and vMIP-I. A second HIV-1 isolate, P1019, also consistently replicated in the presence of AMD3100, albeit at low levels. AMD3100-resistant replication by each of these viruses was completely inhibited upon pre-treatment with vMIP-I and AMD3100 together. Although the HIV-2 strain ETP demonstrated an ability to infect both adult and foetal astrocytes upon the expression of CD4 (Fig. 4.4), it was unable to exploit an alternative coreceptor on Δ32/Δ32 PBMCs. SIVman4 had the capacity to infect CD4+ Leydig cells, adult and foetal astrocytes, as well as CD4- adult astrocytes (Figs. 4.1, 4.4 and 4.5). SIVman4
was also able to infect PBMCs via an alternative coreceptor. Replication of this T-cell line adapted strain was unaffected by AMD3100, vMIP-I and I309, whether alone or in combination. These results therefore show that the HIV-1 and HIV-2 strains that were able to infect astrocytes and BMVECs were also able to exploit an alternative coreceptor on PBMCs, a major target for HIV in vivo.

4.2.8 Δ32/Δ32 CCR5 PBMC infection is not mediated via CCR8 or CXCR6

It has been previously reported that CCR8 and CXCR6 can be exploited by some primary HIV-1 isolates for infection of primary cells in vitro (Lee et al., 2000; Zhang, Y. et al., 1998b).

![Graphs showing RT levels in PBMCs infected with HIV-1 HAN-2 and HIV-2 TER with and without CCR5 and CXCR6 inhibitors.]

Fig. 4.8. Δ32/Δ32 CCR5 PBMC infection is not mediated via CCR8 or CXCR6. Δ32/Δ32 CCR5 PBMCs were preincubated with media alone, AMD3100, I309, CXCL16, I309 + AMD3100 or CXCL16 + AMD3100 at 1000 nM each for 1 hour before infecting with an equal volume of HIV-1 HAN-2 (A) or HIV-2 TER (B). Supernatant was removed every three days up to day 18, and virus production was measured by RT ELISA.
I therefore determined the sensitivity of Δ32/Δ32 PBMC infection by the HIV-1 strain HAN-2 and the HIV-2 isolate TER to inhibition with CCR8 and CXCR6 ligands. In this experiment, replication of both isolates was unaffected by AMD3100 (Fig. 4.8). In addition, both HAN-2 and TER replicated equally as well in the presence of 500 nM of I309 (CCR8 inhibitor) or CXCL16 (CXCR6 inhibitor), either alone or in combination with AMD3100 (Fig. 4.8). So although CCR8 and CXCR6 are responsible for Δ32/Δ32 PBMC infection by some primary isolates, the strains HAN-2 and TER do not use these receptors.

4.2.9 Analysis of HIV/SIV receptor expression on untransformed cell types

To determine the pattern of HIV coreceptor expression, RT-PCR was carried out on mRNA extracted from the three astrocyte cultures, BMVECs, Δ32/Δ32 PBMCs and primary macrophages. The presence of RNA was confirmed in all mRNA preparations by detection of mRNA for the house-keeping gene GAPDH (Fig. 4.9A). CD4 was strongly expressed in Δ32/Δ32 PBMCs and macrophages. In contrast to previous reports, BMVECs and the adult astrocyte cultures 001A and 004.2 were weakly positive for CD4, thus explaining both the inhibition with anti-CD4 antibodies, and infection with the CD4-dependent HIV-1 strain GUN-1v (Figs. 4.2B, 4.6B and 4.9B) (Mukhtar et al., 2002).

A truncated CCR5 transcript was detected in Δ32/Δ32 PBMCs and macrophages, but CCR5 (either truncated or full-length) was not found in any of the other cell cultures tested. CXCR6 expression was detected in PBMCs only, but CXCR4 mRNA was found in Δ32/Δ32 PBMCs and macrophages, as well as in the foetal astrocyte culture F01. CCR3 and CCR8 were detectable on Δ32/Δ32 PBMCs after the number of PCR cycles was increased from 25 to 35 cycles (Fig. 4.9C). All other coreceptors screened for, including CCR3, CCR8, GPR1 and GPR15, were negative on all other primary cell cultures after 25 and 35 cycles (Fig. 4.9C). The orphan receptor RDC1 was detected in Δ32/Δ32 PBMCs, foetal F01 astrocytes and weakly in adult 004.2 astrocytes. In contrast to other studies, the parental NP2/CD4 cell line was also positive for this receptor (Shimizu et al., 2000). None of the known HIV/SIV coreceptors tested here were expressed on the BMVEC and astrocytes cultures able to support infection with HIV/SIV via an alternative coreceptor, as determined by mRNA expression. It is thus concluded that an as yet unidentified receptor for vMIP-I is
partially (astrocytes) or wholly (BMVECs and PBMCs) responsible for infection of the untransformed cell cultures investigated here.

Figure 4.9. mRNA expression of HIV and SIV receptors and coreceptors in primary cell types. RT-PCR was used to test for chemokine receptor mRNA expression. Amplification of GAPDH served as a control for intact mRNA (A). The presence of chemokine receptor mRNA in Δ32/Δ32 CCR5 PBMCs and macrophages, astrocytes and BMVECs was determined, with mRNA from coreceptor expressing cell lines NP2/CD4 (CCR5, CXCR4, CCR3, CCR8, and GPR1) and GHOST (CXCR6 and GPR15) acting as positive controls (B). NP2/CD4 cells naturally expressed RDC1. Primers used are shown in Table 2.2. MWM represents a 100 bp molecular weight DNA ladder. Each experiment was carried out with negative (-; water) and positive controls (+; mRNA from cells expressing the specific receptor), as well as the parental NP2/CD4 cells as a cellular negative control. The absence of contaminating genomic DNA was confirmed by additionally carrying out all PCR reactions on untranscribed mRNA (data not shown).
4.3 Discussion

A wide range of chemokine receptors and related 7TM GPCRs can support infection of indicator cell lines *in vitro*. Many HIV-2 and SIV strains are particularly promiscuous with regards to coreceptor use, but several HIV-1 strains can also use alternative coreceptors. Although many of these are expressed on several CD4$^+$ primary cell types, their use *in vivo* is thought to be insignificant (Albright *et al.*, 1999; Farzan *et al.*, 1997a; Sharron *et al.*, 2000). This clearly demonstrates discordance in coreceptor use *in vitro* and *in vivo*. I analysed the susceptibility of CXCR4$^+$CCR5$^+$ primary cells, including Leydig cells, astrocytes, BMVECs, and Δ32/Δ32 CCR5 PBMCs with CXCR4 blocked by a small molecule inhibitor, to infection with HIV and SIV, and have demonstrated the existence of a previously unknown coreceptor.

4.3.1 Expression of alternative coreceptors on Leydig cells

One of the major routes of transmission of HIV is via sexual contact. Indeed, HIV particles, proteins and/or proviral DNA have been identified in the semen of HIV-positive individuals, in testicular germ cells, of all stages of differentiation, and surrounding Sertoli cells (Baccetti *et al.*, 1994; Baccetti *et al.*, 1998; Borzy, Connell, and Kiessling, 1988; Ho *et al.*, 1984; Krieger *et al.*, 1991; Muciaccia *et al.*, 1998; Shevchuk, Nuovo, and Khalife, 1998; Vernazza *et al.*, 1994). Although these reports demonstrate the presence of HIV proteins at localised sites within the testes, the precise source of seminal HIV particles remains unclear. Testes from AIDS patients display many histological abnormalities, including an arrest in germ cell maturation and a thickening of seminiferous tubule walls (Chabon, Stenger, and Grabstald, 1987; De Paepe *et al.*, 1989). In addition, the testosterone-producing Leydig cells exhibit decreased cell numbers, atrophy and abnormalities in testosterone production (Christeff *et al.*, 1992; Dobs *et al.*, 1988; Shevchuk, Nuovo, and Khalife, 1998). Thus, the localisation of Leydig cells next to testicular blood vessels makes them vulnerable to infection by a blood-borne virus such as HIV.

Consistent with other reports, all HIV-1 isolates tested irrespective of cellular tropism were unable to infect Leydig cells even after expression of CD4 via an adenovirus vector (Shevchuk, Nuovo, and Khalife, 1998). However, a subset of HIV-2 and SIV strains were able to replicate in these Leydig cells in the presence or absence of CD4 expression, albeit to a lower level. Leydig cell infection was unaffected by the chemokines RANTES (CCR5) and eotaxin (CCR3), consistent with previous reports.
demonstrating the absence of these coreceptors (Habasque et al., 2002; Willey et al., 2003b). The inhibitory effects of AMD3100 (a small molecule antagonist of CXCR4) and Q4120 (a CD4 mab), and the low-level expression of these two receptors on these cell cultures, could be explained by the presence of contaminating macrophages (<1%)(Willey et al., 2003b). However, a low level of viral replication (~20%) was still observed in the presence of the CXCR4 and CD4 inhibitors. In addition, virus isolates able to exploit CCR5 or CXCR4 were unable to productively infect these cultures. Moreover, SIV strains that infected Leydig cells use CXCR4 inefficiently at best, supporting the idea that Leydig cells, and not macrophages, are responsible for the observed infection. Due to the scarcity of testicular tissue, samples from different donors were used for infectivity, inhibition and RT-PCR experiments. As such, it is possible that a heterogeneous chemokine receptor expression profile may explain the variable results obtained. However, taken together, these observations indicate the expression and use of a functional alternative coreceptor on Leydig cells able to support HIV-2 and SIV infection.

The coreceptor expressed and exploited on Leydig cells has not been conclusively identified. However, the presence of mRNA for the orphan coreceptor GPR1 in these cultures, in addition to the fact that all isolates able to replicate in Leydig cells cultures can exploit this coreceptor, implicates GPR1 as a possible candidate coreceptor for HIV-2 and SIV infection of Leydig cells (Table 2.7)(Willey et al., 2003b). The in vivo relevance of productive HIV-2 and SIV infection of Leydig cells in vitro remains to be determined; however, a further in-depth study making use of HIV-1 strains with broader coreceptor requirements, or Leydig cells isolated from HIV+ individuals with testicular abnormalities, may identify an HIV-1 subset able to infect Leydig cells and provide a means to examine the possible role(s) of Leydig cell infection in the testicular dysfunction of HIV-infected individuals.

4.3.2 The role of alternative coreceptors in neuropathogenesis

The brain becomes colonised by HIV-1 in approximately 80% of infected individuals during the initial stages of infection, and around one-third of these go on to develop severe neurological disorders classified as AIDS dementia complex (ADC)(section 1.2.3.3)(Lipton, 1994). The main cellular target for HIV within the brain is monocyte-derived microglial cells and perivascular macrophages, but there is evidence for infection of other cell types, such as astrocytes and BMVECs (Moses et
al., 1993; Tornatore et al., 1994a; Williams et al., 2001). Although previous reports indicate only a restricted infection of astrocytes, more recent reports have shown productive infection (Canki et al., 2001; Messam and Major, 2000; Saito et al., 1994; Takahashi et al., 1996; Willey et al., 2003a). The use of p24 (CA, a late gene product) as a marker for HIV-1 infection in this study shows that the in vitro astrocyte infection observed is not restricted to the early phase of viral replication.

Brain resident macrophages and microglia are predominantly infected via CCR5, despite the expression of other coreceptors such as CCR3, and as such, R5 viruses present early in infection predominate in the brain (Albright et al., 1999; Berger, Murphy, and Farber, 1999; He et al., 1997; Shieh et al., 1998). Other cell types within the CNS susceptible to HIV infection are known to be very heterogeneous, and their chemokine receptor expression profiles are therefore controversial (Dorf et al., 2000; Garlanda and Dejana, 1997). The chemokine receptor profile of astrocytes also differs with age; foetal astrocytes express CXCR4 whereas adult astrocytes do not (Reeves et al., 1999). I therefore analysed primary untransformed BMVEC cultures and astrocytes established from adult temporal lobectomies, as well as astrocytes derived from foetal tissue, for expression of a functional HIV coreceptor.

BMVECs and the two adult astrocyte cultures 001A and 004.2, expressing CD4 via an adenovirus vector, were resistant to infection by HIV and SIVs that predominantly utilise CCR5 or CXCR4 alone, despite reports demonstrating expression of CXCR4 on human, and CCR5 on rhesus BMVECs (Edinger et al., 1997b; Mukhtar et al., 2002). The lack of inhibition of BMVEC infection by CCR5 and CXCR4 ligands (as previously reported by Mukhtar et al) further confirms that neither CCR5 nor CXCR4 are expressed on this cell type, and that these coreceptors are not responsible for BMVEC infection (Mukhtar et al., 2002). However, the BMVECs and adult astrocytes were susceptible to infection with a subset of R5X4 HIV and SIV strains. Although these cells are widely accepted as being CD4-negative, the ability of primary HIV-1 isolates (HAN-2 and P1019) to infect even in the absence of adeno-CD4 treatment, as well as inhibition by two anti-CD4 mabs and detection of low-level CD4 expression by RT-PCR, indicates that both these adult astrocytes and the BMVECs do in fact express CD4. Consistent with other studies demonstrating the expression of CXCR4 on foetal astrocytes, the culture FOI was sensitive to infection by X4 and R5X4 viruses upon CD4 expression.
GUN-1v infection of BMVECs was extremely sensitive to inhibition by vMIP-I (an HHV8-encoded chemokine binding CCR8, GPR1 and CXCR6). However, the inability of ligands to CCR8 (I309) and CXCR6 (CXCL16), plus the absence of mRNA expression of these receptors (as determined by RT-PCR), suggests that an as-yet unidentified receptor for vMIP-I is important for infection of these cells. In contrast to BMVECs, astrocyte infection was much less sensitive to vMIP-I. In addition to vMIP-I, the promiscuous chemokines RANTES and eotaxin reduced astrocyte infection. Both foetal and adult astrocytes used here were consistently negative for CCR5 expression, despite several groups reporting CCR5 expression on adult and foetal astrocytes in vivo by in situ immunostaining (Boutet et al., 2001; Dorf et al., 2000; Klein et al., 1999). The lack of inhibition by the CCR5 small molecule inhibitor TAK-779 and the CCR5-specific mab 2D7 confirm that the inhibition by RANTES was not due to the presence of CCR5 on these cells. Inhibition of astrocyte infection by several chemokines (vMIP-I, RANTES and eotaxin) implicates a role for this subclass of receptor. However, only weak inhibition was achieved by each, implying that these chemokines are only minor ligands for the receptor(s) responsible for astrocyte infection. The lack of complete inhibition by vMIP-I suggests that either the vMIP-I receptor implicated for the infection of BMVECs is not solely responsible for astrocyte infection, or that vMIP-I only weakly inhibits this receptor as expressed on astrocytes.

The main source of HIV in the brain is from cellular reservoirs such as infected macrophages/microglial cells, but the mechanism of virus entry into this immunoprivileged site remains unclear. The infiltration of infected monocytes through the blood:brain barrier (BBB), the first line of defence for the brain against HIV and other infectious particles, or macropinocytotic entry of HIV into BMVECs, are suggested mechanisms of HIV entry into this protected compartment (Liu et al., 2002; Persidsky et al., 1999; Persidsky et al., 1997). As BMVECs and astrocytes, the major cell types found in the BBB, possess the capacity to support HIV infection, their infection in vivo would provide a logical route into the brain (Joseph, Lublin, and Knobler, 1997; Mukhtar et al., 2002; Mukhtar and Pomerantz, 2000; Rubin and Staddon, 1999). My results demonstrating that R5 viruses, present early in infection and predominant in the brain, cannot infect BMVECs or astrocytes do not support this model (Willey et al., 2003a). Direct infection of BMVECs resident in the BBB is therefore unlikely to be a significant route of entry of HIV into the brain. However, that infection of these cells by broadly-tropic viruses during the later stages of disease may
contribute to the breakdown of the BBB and HIV neuropathogenesis, cannot be excluded. Moreover, the alternative coreceptor demonstrated here on CCR5' cultured astrocytes may potentially confer infection of astrocyte subsets critical for brain homeostasis, and thus impact on neuropathology (section 1.2.3.3).

4.3.3 The use of alternative coreceptors on major target cells of HIV and SIV in vivo

The exploitation of alternative coreceptors (other than CCR5 or CXCR4) for HIV infection of astrocytes and BMVECs supports a role for such coreceptors; however, these cells are not the major targets of HIV-1 in vivo. There is evidence supporting the use of alternative coreceptors on PBMCs in vivo (Lee et al., 2000; Sharron et al., 2000; Zhang, Y. et al., 1998b). In this study I found that several of the HIV-1 and HIV-2 isolates able to infect BMVECs and astrocytes were also able to replicate in PBMCs that lack CCR5 and with CXCR4 blocked by AMD3100. A critical point to mention is that residual replication of the X4 isolate 2044 in the presence of AMD3100 was observed in some experiments. vMIP-I completely inhibited replication of the other isolates (such as HAN-2 and P1019) confirming that replication in the presence of AMD3100 is not due to virus variants resistant to AMD3100 but retaining use of CXCR4. Moreover, it has been demonstrated that vMIP-I does not interact with or influence HIV infection via CXCR4 (Dairaghi et al., 1999; Simmons et al., 2000).

As seen with BMVEC infection, AMD3100-resistant PBMC replication was very sensitive to vMIP-I inhibition, confirming that an alternative coreceptor is active. As mentioned above, vMIP-I inhibits virus replication via CCR8, GPR1 and CXCR6 (Dairaghi et al., 1999; Endres et al., 1999; Simmons et al., 2000). All R5X4 HIV/SIVs able to infect BMVECs, astrocytes and PBMCs could efficiently exploit both CCR8 and GPR1 for infection of CD4⁺ indicator cell lines. However, although both CCR8 and CXCR6 were detected these Δ32/Δ32 PBMCs by RT-PCR, their ligands (I309 and CXCL16, respectively) had no effect on viral replication, either alone or in combination with AMD3100. mRNA for GPR1 was not detected on these cells. These data strongly imply an as yet unidentified vMIP-I receptor. An alternative, albeit less likely, explanation is that vMIP-I induces down-modulation of another receptor, or intracellular signalling events that negatively affect viral replication. However, previous reports show that vMIP-I has no effect on signalling via many chemokine receptors expressed on PBMCs, including CCR2, CCR4, CCR5, CCR7, CXCR2, or CXCR4 (Dairaghi et al., 1999). Thus, a currently unidentified vMIP-I receptor is
expressed and functional as a coreceptor on PBMCs, a major cell target for HIV infection in vivo.

4.4 Summary

I have demonstrated that an unknown alternative coreceptor is expressed and used on several primary cell types, importantly including PBMCs, a major target of HIV in vivo. The identity of this coreceptor remains to be conclusively elucidated. A hallmark of the BMVEC and PBMC infection via an alternative coreceptor demonstrated here was the high sensitivity to inhibition by vMIP-I. To date, vMIP-I has been reported to inhibit HIV infection mediated through CCR8 (Dairaghi et al., 1999; Endres et al., 1999) as well as GPR1 and CXCR6 (Simmons et al., 2000). GUN-1v and the other HIV and SIV strains identified here by their capacity to use alternative coreceptors were able to use both CCR8 and GPR1 for infection of indicator cell lines, in addition to CCR5 and CXCR4. Furthermore, GUN-1v has previously been reported to use alternative coreceptors for infection of astroglial U87 cells, primary mesengial kidney cells as well as BT cells. CCR8 was implicated as a coreceptor for BT infection (Jinno et al., 1998) and GPR1 for mesengial cell infection (Tokizawa et al., 2000); however, inhibition by ligands to these coreceptors was not shown. My observations that the CCR8 ligand I309 had no effect on the replication of any HIV or SIV isolate in BMVECs, astrocytes or Δ32/Δ32 PBMCs, implies that vMIP-I is able to inhibit HIV infection of PBMCs via another chemokine receptor. Thus, a novel unknown receptor for vMIP-I is capable of supporting infection of a range of primary cell types by a small subset of HIV and SIV isolates.
CHAPTER 5

Comparison of HIV-1 subtype B and C coreceptor use and cell tropism

5.1 Introduction

The major HIV-1 group M is split into 11 genotypically distinct subtypes (section 1.1.3). Of these, subtype C is the most prevalent, accounting for over 50% of global infections, predominantly in India and Africa (Cassol et al., 1996; Cecilia et al., 2000; Dietrich et al., 1993; UNAIDS/WHO, 2002a). It is well documented that approximately 50% of subtype B isolates undergo a switch in biological phenotype (M-tropic to T-tropic) (De Jong et al., 1996; De Wolf et al., 1994; Trkola et al., 1998; Zhang et al., 1997). In these cases, CCR5-using viruses evolve into rapidly replicating variants that exploit CXCR4, and potentially other coreceptors, in addition to, or instead of CCR5 (Chapter 4) (Asjo et al., 1986; Bjorndal et al., 1997; Cheng-Mayer et al., 1988; Schuitemaker et al., 1992; Tersmette et al., 1988). This switch in viral phenotype has been observed in all HIV group M subtype infections; however, surprisingly few CXCR4-using subtype C viruses have been isolated (Abebe et al., 1999; Bjorndal et al., 1999; Peeters et al., 1999; Ping et al., 1999; Tien et al., 1999; Tscherning et al., 1998; Zhang, H. et al., 2002).

The coreceptor switch from CCR5 to CXCR4 in subtype B-infected individuals correlates with a rapid decline in CD4+ T-cell numbers and an increase in viral load, and thus disease progression (Koot et al., 1992; Penn et al., 1999; Richman and Bozzette, 1994; Schellekens et al., 1992). Individuals infected with subtype C often carry high viral loads, have a low CD4+ T-cell count throughout infection, and thus frequently undergo rapid disease progression (Kanki et al., 1999; Mehendale et al., 2002; Neilson et al., 1999). Thus, the biological phenotype of subtype C infection does not appear to correlate with that of subtype B. Evolution of most HIV-1 subtypes to exploit CXCR4 for infection is associated with characteristic sequence changes in the coreceptor-determining V3 loop of gp120 (Chesebro et al., 1992; Fouchier et al., 1992; Peeters et al., 1999; Xiao et al., 1998b; Zhang et al., 1997). For example, X4 strains generally have more basic residues at key locations (11 and 25) within the V3 loop, resulting in an increase in the net positive charge of the V3 e.g. CTRPNNNTRKSIITIGPGRAYATGDIPQDIRQAHC (+3) for the R5 SF162, and
CTRPNNNTRKRIIRQGPGRAFVTIGKIGNMRQAHC (+9) for the X4 strain HXB2 (section 1.7.3)(De Jong et al., 1992; Milich, Margolin, and Swanstrom, 1993; Shioda, Levy, and Cheng-Mayer, 1992). Extensive analysis of subtype C V3 loops indicates that this region is of a low positive charge, consistent with sequences from R5 isolates, and are relatively conserved (Cilliers et al., 2003; Gaschen, Korber, and Foley, 1999; Korber et al., 1994; Ping et al., 1999; Treurnicht et al., 2002). However, it is important to remember that this coreceptor switch only occurs in 50% of subtype B infections, yet the majority of HIV+ patients eventually progress to AIDS. The evolution of CXCR4-using isolates is therefore neither sufficient for, nor a prerequisite, for progression to AIDS (de Roda Husman et al., 1999).

Although CCR5 and CXCR4 are major HIV coreceptors, other chemokine receptors and related 7TM GPCRs support infection of CD4+ indicator cell lines. Moreover some primary cell types, including PBMCs, support infection by a subset of subtype B isolates in vitro via an unknown coreceptor (Chapter 4)(Willey et al., 2003a). To date, subtype C isolates are almost exclusively R5-using; however, extensive analyses of their coreceptor use beyond CCR5 and CXCR4 have not been done. One possibility is that subtype C viruses exploit other coreceptors more efficiently than subtype B's. Thus during disease progression, subtype C's may undergo an expansion in coreceptor use to include other coreceptors, but not CXCR4. Here, a large panel of primary subtype B and C isolates were compared for their ability to exploit an alternative coreceptor to infect primary, untransformed CCR5+ and CXCR4+ BMVECs and adult astrocytes, demonstrated in Chapter 4 to support infection by HIV-1 (Chapter 4)(Willey et al., 2003a). Isolates identified to infect such cells, in addition to several unable to infect these cells, were subjected to extensive analysis of their coreceptor usage properties. The V3 sequences of some of these subtype B and C isolates were determined, in order to ascertain the presence of residues that correlate with the capacity to exploit alternative coreceptors. The capacity of these subtype C strains to replicate on PBMCs negative for CCR5 was also assessed.
5.2 Results

5.2.1 Use of an alternative coreceptor on primary human cells by HIV-1 subtype C isolates

As subtype C viruses are reported to rarely use CXCR4, the titres of 34 primary subtype C isolates on stable NP2/CD4 cells expressing either CCR5 or CXCR4 were determined. Details of these isolates, including the country of origin, patient information, and the relevant Genbank and literature references, are detailed in Table 2.8. With the exception of two isolates (96USNG31 and CM9), all of the subtype C strains tested here (94%) used CCR5 but not CXCR4 (Table 5.1). As individuals infected with subtype C still rapidly progress to AIDS, I tested their capacity to infect CCR5- and CXCR4-negative primary, untransformed human cell types demonstrated in Chapter 4 to support infection via an alternative coreceptor. Out of 34 strains, two (97USNG30 and CM9) were able to infect both brain microvascular endothelial cells (BMVECs) and adult astrocytes (001A). An additional seven strains (96USNG58, 97/ZA/009, 98IN022, 98CN009, 97TZ017, MJ4 and CM4) were able to infect one cell type, albeit to low levels (Table 5.1). Of these 9 strains, 8 were R5 only, and only one (CM9) was R5X4, although 97USNG30 did evolve to exploit CXCR4 after a single passage through human PBMCs.

5.2.2 Alternative coreceptor use on primary cells by subtype B strains

In order to reliably compare the coreceptor-usage patterns between HIV-1 subtype B and subtype C isolates, the titres of a panel of primary and molecularly cloned subtype B strains (as described in Table 2.7) were determined on CCR5+ or CXCR4+ cells stably expressing CD4. Unlike the subtype C panel, which was remarkably R5-tropic, many of the subtype B's tested were able to exploit CXCR4 alone, or in addition to CCR5. Out of 27 isolates, 14 (51.8%) were R5, 5 (18.5%) were R5X4 and 8 (29.7%) were X4 only (Table 5.2). Thus, approximately 50% of this subtype B panel is able to use CXCR4 for infection. Of the 27 isolates tested, 7 could infect BMVECs and/or astrocytes. Four of these were R5X4, following observations that the expansion in coreceptor use from R5 to R5X4 confers the capacity of some HIV isolates to infect CCR5 and CXCR4-negative cells (Chapter 4). However, the use of this larger panel of isolates resulted in the identification of a further three subtype B strains (BR65, SL-8 and 93US143) that were able to infect these cells, despite only being able to use CXCR4 on indicator cells.
Table 5.1. Use of an alternative coreceptor by a panel of subtype C isolates. Titres of a panel of subtype C isolates on CD4-positive CCR5- and CXCR4-expressing indicator cell lines were determined. The capacity of these isolates to exploit an alternative coreceptor to infect primary BMVECs and adult astrocytes was then analysed. A value of 0 represents <10 ffu/ml. All isolates are primary patient strains, unless indicated. * indicates a molecular clone.
Table 5.2. Use of an alternative coreceptor on primary cells by a panel of subtype B strains. Titres of a panel of subtype B strains were determined on CD4⁺CCR5⁺ and CD4⁺CXCR4⁺ cell lines. The capacity of these isolates to infect primary BMVECs and adult astrocytes via an alternative coreceptor was also determined. A value of 0 represents <10 ffu/ml.

5.2.3 Analysis of subtype B and C coreceptor use

Section 5.2.1 demonstrates that a subset of subtype C strains (mainly unable to exploit CXCR4) can infect primary cell types not expressing CCR5 or CXCR4 (Table 5.1). In comparison, all the subtype B isolates able to infect these cells can exploit CXCR4, either alone or in combination with CCR5 (Table 5.2). To identify a pattern
between the capacity to infect these cells and coreceptor requirements, all subtype B and C isolates able to infect BMVECs and/or astrocytes, in addition to some unable to infect these cells, were analysed for their capacity to infect cell lines stably expressing a range of alternative coreceptors (Table 5.3, subtype C; Table 5.4, subtype B).

<table>
<thead>
<tr>
<th>Virus</th>
<th>NP2/CD4</th>
<th>*NP2/CCR5</th>
<th>CCR3</th>
<th>CCR8</th>
<th>APJ</th>
<th>GPR1</th>
<th>GPR15</th>
<th>CXCR6</th>
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<tr>
<td>97USNG30</td>
<td>0</td>
<td>0</td>
<td>6.3 x 10^3</td>
<td>3.3 x 10^2</td>
<td>50</td>
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<td>1.1 x 10^2</td>
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</tr>
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<td>0</td>
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<td>2.8 x 10^2</td>
<td>0</td>
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<td>40</td>
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<td>4.6 x 10^2</td>
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Table 5.3. Analysis of subtype C coreceptor use. The capacity of a panel of subtype C isolates to exploit a range of alternative HIV/SIV coreceptors was determined. Stable NP2/CD4 cells expressing a range of coreceptors was used, except where indicated. * = CD4-negative NP2/CCR5 cells; # = GHOST cell line. nd = not determined. Isolates in bold (upper half) are able to infect BMVECs/astrocytes; isolates in normal text are unable to infect these cells. 0 represents <10 ffu/ml. Infectivity data on NP2/CD4/CCR5 and NP2/CD4/CXCR4 cell lines is shown in Table 5.1.

None of the isolates (either subtype B or C) were able to infect the NP2 cell line in the absence of coreceptors, or NP2/CCR5 cells lacking CD4 (Tables 5.3 and 5.4). Some of the subtype C strains that infected BMVECs and/or astrocytes to low levels (98/CN/009, MJ4, and 98/TZ/017) were unable to exploit any of the alternative coreceptors tested here, while other isolates unable to infect BMVECs/astrocytes (PCPl and 92BR025) had a broader coreceptor usage which included CCR3, CCR8 and
CXCR6 (Table 5.3). As observed with the panel of subtype C strains, not all subtype Bs able to infect BMVECs and/or astrocytes are broadly tropic. For example, BR65 infected the adult astrocytes 001A (albeit weakly), yet did not infect cells by any coreceptor other than CXCR4 (Table 5.2). Likewise, some strains (BR92 and 2053) could utilise CCR3 and CCR8, yet could not infect BMVECs and astrocytes. One common observation between the subtype B and C strains able to infect BMVECs/astrocytes was the ability of several to exploit APJ, GPR1, or GPR15 for infection of cell lines. In contrast, use of CCR3 and CCR8 did not correlate with infection of the CCR5- and CXCR4-negative cell types tested here.

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<th>CCR8</th>
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Table 5.4. Coreceptor-usage properties of subtype B strains. The coreceptor-usage properties of isolates able (bold text) or unable (normal text) to infect BMVECs/astrocytes was determined. Coreceptor-positive NP2/CD4 cells were used, except where indicated. * = CD4-negative NP2/CCR5 cells; b = GHOST cells. nt = not tested. Isolates in bold (upper half) are able to infect BMVECs/astrocytes; isolates in normal text are unable to infect these cells. A value of 0 represents <10 ffu/ml. Infectivity data on NP2/CD4/CCR5 and NP2/CD4/CXCR4 cell lines is shown in Table 5.2.

5.2.4 Replication of subtype C isolates in wt/wt versus A32/A32 CCR5 PBMCs

As some CCR5-only subtype C strains were able to infect CCR5-negative cells, their capacity to infect human PBMCs with (wt/wt) and without (A32/A32) CCR5 was compared. No correlation was found between capacity to infect BMVECs/astrocytes, the capacity to exploit alternative coreceptors, or the ability to replicate in PBMCs lacking CCR5.
Figure 5.1. Replication of HIV-1 subtype C in wt/wt versus Δ32/Δ32 CCR5 PBMCs. The replicative capacity of a subset of HIV-1 subtype C strains in Δ32/Δ32 CCR5 (red line) and wt/wt (black line) PBMCs was compared. Supernatant was removed every 3 days, and virus production measured by RT ELISA. All points are an average of triplicate samples, and results are representative of two independent infections.
Out of 19 subtype C strains tested, 6 (97USNG30, CM4, CM9, PCP1, TS/347/TB5, 97/ZA/003) replicated to some extent in Δ32/Δ32 PBMCs, yet only one of these (CM9) uses CXCR4. Therefore, although the vast majority of subtype C strains are strongly R5 and non-X4-using, a small percentage is able to infect CCR5-negative PBMCs via an alternative coreceptor, albeit to a low level in most cases.

5.2.5 Subtype C infection of Δ32/Δ32 CCR5 PBMCs is mediated via an alternative coreceptor

Previous results indicated that infection of these Δ32/Δ32 PBMCs by some HIV-1 and HIV-2 isolates was sensitive to inhibition with vMIP-I in conjunction with AMD3100. I therefore investigated the capacity of vMIP-I to inhibit subtype C replication in Δ32/Δ32 CCR5 PBMCs.

![Graphs of CM4, CM9, and 97USNG30 infection inhibition](image)

**Figure 5.2. Inhibition of Δ32/Δ32 PBMC infection by HIV-1 subtype C.** Δ32/Δ32 CCR5 PBMCs were preincubated with media alone, AMD3100, vMIP-I or AMD3100 + vMIP-I at 100 nM each, before infecting with the HIV-1 subtype C strains CM4, CM9 or 97USNG30. Supernatant was removed every 3 days up to day 18, and virus production measured by RT ELISA.
Infection by the strain CM9, which efficiently uses CXCR4, was inhibited by the CXCR4 inhibitor AMD3100, although it did escape inhibition after approximately 9 days in culture (Fig. 5.2). vMIP-I, which inhibits infection via CCR8, CXCR6 and GPR1, had no effect on the replication of this virus on its own, yet completely eliminated residual infection when used in addition to AMD3100. Although neither uses CXCR4, infection by both CM4 and 97USNG30 was reduced by AMD3100 (approximately 50% and 65% reduction, respectively). vMIP-I also inhibited both these isolates by 50%. As observed with CM9 and the HIV-1 subtype B strain HAN-2 (Chapter 4), infection with 97USNG30 was completely blocked in the presence of AMD3100 and vMIP-I together; however, CM4 was still able to replicate with these inhibitors. Thus, as observed with HIV-1 subtype B and HIV-2, AMD3100-resistant infection of Δ32/Δ32 CCR5 PBMCs by some subtype C viruses is mediated via a vMIP-I receptor. However, this is not the case with all isolates, as demonstrated by the broadly tropic isolate CM4.

5.2.6 Analysis of subtype B and C gp120 V3 sequences

The V3 loop of HIV gp120 is the predominant region determining R5 or X4 use (section 1.7.3). Most of these subtype C strains were R5 using, yet some were able to infect CCR5-negative cells. Their V3 loops were therefore analysed for the presence of specific residues or sequence motifs that might account for both their coreceptor use and ability to infect CCR5'CXCR4' cells. CM9 is one of the few subtype C isolates to use CXCR4, and to efficiently infect astrocytes, BMVECs and Δ32/Δ32 PBMCs in the presence of CXCR4 inhibitor, and it possessed several differences within its V3 loop. The residues situated at positions 11 [generally serine (S)] and 25 [generally aspartic acid (D) or glutamic acid (E)] are frequently substituted for basic amino acids in X4-using isolates. Indeed, CM9 had substitutions at both these locations, with an arginine at position 11 and a lysine at position 25, contributing to the high positive charge of this isolate (Table 5.6, bold text). All other subtype C, and B strains sequenced had a neutral amino acid (serine or glycine) at position 11. Again at position 25, most of the subtype B and C's sequenced possess the consensus acidic residues. The subtype B strains HAN-2 and GUN-1v both had a basic amino acid at position 25, and like CM9, these strains were very efficient in their infection of BMVECs, astrocytes, and cell lines via GPR1 (Tables 5.1, 5.2, 5.3 and 5.4). In addition, the subtype C isolates 97/ZA/009, 93/MW/965 and 92/BR/004 all contain substitutions at this location, but only with
neutral residues (glycine or alanine). CM4 possesses a valine at position 25, which increases the overall number of hydrophobic amino acids in this V3 loop to 9.

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<th>Charge</th>
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Table 5.5. **Subtype B and C V3 loop sequence analysis.** * indicates sequences from Genbank. Residues that do, or may, play a role in coreceptor use are highlighted. The V3 tip sequence (GXGX) is underlined. Bold = residues often changed in X4-using viruses; highlighted grey = proline residues potentially altering V3 helix formation; highlighted in red = hydrophobic residues, possibly altering V3 conformation. Potential N-linked glycosylation sequences (N-X-S/T) are highlighted in blue.

The hydrophobicity of a protein often influences the overall conformation. All subtype C envelopes had between 7 and 8 hydrophobic amino acids within the V3 loop, indicating that variation of the hydrophobicity of this region is not associated with coreceptor use, or ability to exploit an alternative coreceptor on primary cells (Table
5.5, highlighted in red). However, CM4 has 9 hydrophobic amino acids within its V3 sequence, has a very broad coreceptor use and is able to infect Δ32/Δ32 CCR5 PBMCs in the presence of CXCR4 inhibitors (Table 5.5 and Fig. 5.2). A higher hydrophobicity may thus have some influence on the coreceptor phenotype of this strain. The loss of N-linked glycosylation sites within the HIV envelope influences the CD4-independence and neutralisation sensitivity of HIV and SIV (Edwards et al., 2001; Puffer et al., 2002). Glycosylation sequences within the V3 loop of these isolates were thus also analysed. With the exception of CM9, all subtype B and C isolates sequenced possessed an intact N-linked glycosylation sequence between residues 6-8 (Table 5.5, highlighted in blue). In addition to having two additional basic amino acids, and a higher hydrophobicity to other subtype C V3s, the N-linked glycosylation site was absent in CM9. This may alter the conformation of the V3 loop, enabling it to interact with a broader range of coreceptors. To summarise, there are very few differences between the subtype B and C V3 loop sequences, and there appears to be no clear correlation between V3 loop sequence, and the capacity to exploit alternative coreceptors, to infect BMVECs and/or astrocytes, or the capacity to infect Δ32/Δ32 CCR5 PBMCs.
5.3 Discussion

Approximately 50% of all HIV-1 group M subtypes undergo a broadening of coreceptor use from R5 to R5X4, yet this phenotype appears to be strikingly absent in subtype C. In subtype B, expansion in cellular tropism is often associated with a capacity to use other coreceptors on indicator cell lines or, for a subset of HIV/SIV isolates, on primary cell types (Chapter 4). As subtype C-infected individuals often experience a rapid progression to disease, it is somewhat surprising that X4-using isolates are only rarely isolated. However, it may be the case that subtype C strains undergo an expansion in coreceptor use excluding CXCR4. It is thus possible that the frequency of isolates able to exploit an alternative coreceptor for the infection of primary human cells is higher in subtype C strains than in subtype B. This chapter therefore describes a detailed analysis to compare the frequency of isolates able to exploit functional coreceptors on untransformed human cells within the HIV-1 group M subtype C than subtype B.

5.3.1 Frequency of CXCR4-use in subtype B and C isolates

Out of a panel of 34 primary subtype C isolates, 94% utilised CCR5. Of the two that could use CXCR4 to infect indicator cell lines, both were R5X4 tropic. In comparison, over 50% of 27 primary and TCLA subtype B strains were R5, only 18% were R5X4 yet almost 30% were X4 only. Thus in agreement with published reports, the subtype C isolates tested here infrequently used CXCR4, whereas approximately 50% of the subtype B strains used X4 either alone or in addition to R5.

Subtype C-infected individuals have high plasma viral RNA levels and low CD4+ T-cell counts in comparison to other HIV-1 subtypes, and appear to experience a more rapid progression (Mehendale et al., 2002; Neilson et al., 1999). Thus in the case of subtype C infections, the appearance of more pathogenic virus species and progression to AIDS does not correlate with the use of CXCR4. There are several subtype-specific, genotypic characteristics which may contribute to this apparent increased pathogenicity. Subtype C isolates commonly possess 3-4 recognition sites for the transcription factor NF-kB within the 3' LTR, whereas other subtypes normally possess 2. These additional sites increase transcription from the LTR, which may increase virus production levels and thus the pathogenicity of this subtype (Bjorndal et al., 1999; Gao et al., 1996; Montano et al., 1997; Salminen et al., 1996). In addition, subtype C strains commonly have a truncation in the ORF for Rev, and an insertion
within Vpu, all of which may influence the relative infectivity of released viral particles (Gao et al., 1998; van Harmelen et al., 2001).

Geographic and environmental factors may affect the phenotype and outcome of subtype C infections. Infection with tuberculosis, extremely common in areas where subtype C is predominant, is reported to increase levels of CCR5 expressed on macrophages, and thus may contribute to the high frequency of CCR5-use in this HIV subtype (Cecilia et al., 2000; Cilliers et al., 2003; Clerici et al., 2000; Fraziano et al., 1999; Kurosu et al., 2002). Indeed, virus isolated from late-stage patients with tuberculosis infections was found to be R5 only (Morris et al., 2001). It is also possible that poor health care in areas where subtype C predominates (Africa and India) may result in patient death before late-stage, CXCR4-using virus can emerge, or be isolated and characterised. Some subtype C strains from both Africa and India have been demonstrated to use CXCR4, indicating that there isn’t an absolute block to evolution to CXCR4 use in this subtype (Table 5.1)(Cilliers et al., 2003)(www.aidsreagent.org). Precisely why the use of CXCR4 in this subtype, and in these geographic locations, is rare, is unclear.

All of the subtype C strains analysed here, and all those available at the NIH Aids Research and Reference Reagent Program, are from asymptomatic patients. As CXCR4-using variants tend to emerge during later stages of disease, it is not necessarily surprising that subtype C is reported to be largely R5-using. A recent publication investigating coreceptor-use of subtype C’s taken from late-stage patients found 17% of patients harbour X4-using, SI isolates (Cilliers et al., 2003). The phenotypic difference observed with subtype C may thus be mimicking that previously observed with subtype E, whereby upon initial discovery it appeared to be R5 only, yet subsequent establishment within the population found X4 strains as commonly within this subtype as in all others (De Wolf et al., 1994; Menu et al., 1999; Utaipat et al., 2002). Thus, although current data imply that CCR5 is the major coreceptor for HIV-1 subtype C, it is crucial that virus from late-stage patients is isolated and characterised in order to gain a true evaluation of coreceptor use within this subtype.
5.3.2 Infection of CCR5- and CXCR4-negative primary cells by subtype B and C strains

The rarity of CXCR4-use and demonstration of rapid disease progression within subtype C-infected individuals may indicate an evolution of virus strains able to utilise alternative coreceptors, aside from CXCR4. Thus, one might expect a higher frequency of such strains able to use an alternative coreceptor within subtype C in comparison to subtype B. Analysis of a large panel of subtype B and C and strains (27 and 34, respectively) found no difference in the frequency of isolates able to infect CCR5\- CXCR4\- primary cells, with 26% of both subtypes able to exploit an alternative coreceptor expressed on BMVECs and/or astrocytes. All of the subtype B strains with this phenotype were either R5X4 or X4, whereas most (8 out of 9) of the subtype C’s were R5 only.

Of the 9 subtype C viruses able to infect BMVECs and/or astrocytes, only 3 (97USNG30, CM4 and CM9) were able to productively replicate in Δ32/Δ32 CCR5 PBMCs. However, as infection of BMVECs/astrocyte was to a very low level with most subtype C strains (including 96USNG58, 98CN009 and 98TZ017), this is not particularly surprising. With the exception of CM4, these strains gave the highest infectivity titres on both BMVECs and astrocytes. Only one of these (CM9) used CXCR4 on indicator cell lines and, as expected, CM9 replication on Δ32/Δ32 CCR5 PBMCs was inhibited by AMD3100. 97USNG30 did evolve to use CXCR4 after a single passage in PBMCs; however, this isolate was still able to replicate in Δ32/Δ32 CCR5 PBMCs in the presence of CXCR4 inhibitors. Thus, as seen with the subtype B strain HAN-2, the capacity of subtype C to infect CCR5\-CXCR4\- primary cells and Δ32/Δ32 CCR5 PBMCs without CXCR4 appears to correlate with tropism expansion to use CXCR4 and an ability to infect BMVECs and/or astrocytes. This is clearly not always the case, as the subtype C strain CM4 could not infect BMVECs/astrocytes or use CXCR4 to infect indicator cell lines, yet it was able to efficiently replicate in Δ32/Δ32 CCR5 PBMCs in the presence of AMD3100.

As seen with HAN-2, a subtype B with a true capacity to exploit an alternative coreceptor to infect primary cells, a broad coreceptor use of these viruses was observed. 97USNG30, CM4 and CM9 were all able to efficiently use other coreceptors, including CCR3, CCR8 and GPR1, to infect indicator cell lines. However, a broad coreceptor use
did not automatically guarantee the capacity to infect CCR5\textsuperscript{+}CXCR4\textsuperscript{-} primary cells, as PCP1 and 92/BR/025 could use CCR3, CCR8 and CXCR6 to infect CD4\textsuperscript{+} indicator cell lines. The same pattern was observed within the subtype B strains, where isolates able to efficiently infect BMVECs, astrocytes and/or Δ32/Δ32 CCR5 PBMCs (HAN-2, P1019 and GUN-1v) could all use CCR3, CCR8 and GPR1 on indicator cell lines. Again, an exception was observed with 2005, which had a narrower coreceptor use.

As seen with HAN-2 (Fig. 4.7), infection of Δ32/Δ32 CCR5 PBMCs with both CM9 and 97USNG30 was sensitive to inhibition with vMIP-I, whereas infection with CM4 was not. Both CM9 and 97USNG30 can use GPR1, one of the known receptors for vMIP-I, whereas CM4 does not. However, mRNA expression of this receptor was not detected on these cells (Fig. 4.9). The identity of the receptor used by these subtype C strains is therefore unknown.

5.3.3 HIV-1 subtype C V3 loop sequence does not correlate with alternative coreceptor use

The V3 loop of the HIV envelope determines whether it uses CCR5 or CXCR4 (section 1.7.3). This region of some of the subtype C strains was sequenced to determine the presence of specific sequence motifs or residues that might confer alternative coreceptor use. As previously reported, the subtype C V3 sequences were very homogeneous (Björndal et al., 1999; Korber et al., 1994; Ping et al., 1999). With the exception of CM9, which was more variable than all other subtype C V3 sequences, all subtype Cs had the characteristic GPGQ sequence at the V3 loop crown, as opposed to GPGR found in subtype B, and most had a low (1-4) positive charge associated with a NSI (R5) phenotype (Cilliers et al., 2003; Gaschen, Korber, and Foley, 1999; Hung, Vander Heyden, and Ratner, 1999; Xiao et al., 1998a; Zhang, H. et al., 2002).

As the coreceptor use of these subtype C strains was variable, ranging from strict R5-use to broad coreceptor-use despite a common V3 loop tip sequence (GPGQ), this region does not fully explain coreceptor usage as previously proposed (McKnight et al., 1995; Thompson, Cormier, and Dragic, 2002). Indeed, studies have demonstrated a role for the V4/V5 loops for R5X4 and X4 phenotypes (Singh and Collman, 2000). In addition, the V1/V2 loops of the envelope have been implicated for alternative coreceptor-use (Hoffman et al., 1998).
Other residues demonstrated to be important for CCR5-use in subtype C, including R298, N301, N302, T303, I322, I325 and R326, were present in all subtype Cs sequenced here, again with the exception of CM9 (Suphaphiphat et al., 2003). This virus strain was able to infect both BMVECs and astrocytes very efficiently, and could also use alternative coreceptors, in addition to CXCR4, very well. It is therefore not surprising that its V3 loop is more diverse than the other isolates examined here. However, as such diversity was only observed in one isolate, specific features in the V3 loop which may confer broad tropism could not be identified.

5.4 Summary

In agreement with other reports, the majority of subtype C isolates investigated here used CCR5 and not CXCR4. However, as all were from asymptomatic individuals, who generally only harbour NSI viruses, the R5-use of subtype C may be an over-represented phenotype. The use of alternative coreceptors on primary cells by subtype C’s is rare and no more common than that seen with subtype B. This phenotype generally correlated with a broadening of coreceptor use, although no specific motifs or substitutions were apparent in the V3 loop sequences. Thus subtype C does not expand its tropism via alternative coreceptors excluding CXCR4. Although only a minority of HIV isolates have been identified that possess the capacity to use this alternative coreceptor, with over 42 million people infected worldwide such minority variants need to be kept in mind. As subtype C is the most common global HIV-1 subtype, it is vital to fully understand its coreceptor use and phenotype, particularly with respect to vaccine development and the introduction of coreceptor inhibitors. Further detailed analysis of subtype C strains from late-stage patients is thus required to fully understand the evolution of coreceptor-use within this HIV-1 subtype.
CHAPTER 6

Conclusions and further work

Current highly active antiretroviral therapy (HAART) regimens have revolutionised the treatment of HIV, but it is not without its caveat. This combination of drugs, targeting the essential retroviral enzymes reverse transcriptase (RT) and protease (PR), dramatically decreases the viral load in an individual and allows the immune response and CD4+ T-cell counts to remain stable, thus increasing not only the quality of life but also the life-expectancy of HIV+ individuals. The success of HIV therapy largely relies upon adherence to a complex regimen requiring multiple administrations of large numbers of tablets, a feat that even patients with a stable lifestyle find difficult to maintain. In addition, the side-effects of many of these medications are often extremely severe. However, the biggest drawback to HAART, as is the case with treatment of many other pathogenic organisms, is the evolution of resistance. The high error rate in HIV genome replication results in a rapid adaptation to any selective pressures present. Thus, the constant presence of RT and PR inhibitors in an individual on HAART eventually selects for variants able to actively replicate and infect new cells even in their presence. Unfortunately, as drugs within a single class [such as non-nucleoside reverse transcriptase inhibitors (NNRTIs)] are often structurally similar, drug-resistant virus populations are frequently resistant to a whole class of inhibitor, rendering a large number of therapy options ineffective. These multi-drug resistant variants pose a huge threat, particularly as such strains are appearing at an alarming rate in newly-infected people.

The need for new HIV therapy is thus urgent. There are several points in the HIV life cycle which could be targeted by novel intervention therapies. For example compounds or proteins that bind to the transmembrane envelope subunit gp41, such as T20 (enfuvirtide), inhibit HIV infection by preventing successful virus:cell membrane fusion. Although the majority of HIV-1 isolates are dependent upon the primary receptor CD4 for infection, there are many HIV-2 and SIV strains that can infect in the absence of CD4, and there is an accumulating amount of evidence for the productive infection of CD4-negative cell types by HIV-1, as shown in this thesis. Thus CD4 itself is not an ideal target for intervention therapies. However, to date there is no firm
evidence demonstrating HIV infection in the absence of a coreceptor, making these molecules a prime target for novel therapeutics. CCR5 may be the most appropriate coreceptor to target, as removal of CCR5 has no significant effect on health or survival of individuals homozygous for a 32-base-pair deletion in CCR5. In addition, CCR5 is used by almost all transmitted virus populations, and such R5 strains persist throughout infection. In contrast, removal of either CXCR4 or its single chemokine ligand SDF-1α is fatal before or shortly after birth, and HIV variants using CXCR4 only evolve during the later stages of disease.

Due to the potential of inducing adverse inflammatory or anti-inflammatory immune responses, as well as the expense of producing large quantities of protein, CCR5-binding chemokines are not suitable as therapeutics even though they are able to inhibit infection by HIV. In this thesis, six small molecule inhibitors of CCR5 developed by Pfizer Global Research and Development, Sandwich, Kent, UK, were analysed. They were found to be extremely potent inhibitors of CCR5-mediated infection by a large range of both R5 and R5X4 HIV strains (both HIV-1 and HIV-2), as well as SIV isolates, on a number of cell types including PBMCs and macrophages (Chapter 3). Such small molecule inhibitors of CCR5, the lead compound of which is currently in Phase II clinical trials, are thus extremely promising new anti-HIV drugs to be used in conjunction with current therapy.

Although CCR5 and CXCR4 are the major HIV coreceptors, there are many other chemokine receptors and related orphan 7 TM GPCRs able to support infection by HIV \textit{in vitro}. Such alternative coreceptors are believed to be insignificant with respect to use by HIV \textit{in vivo}, but may pose a threat when CCR5-specific inhibitors are included in HIV therapy regimens. In Chapter 4 of this thesis I describe an as-yet unidentified coreceptor exploited by a subset of HIV and SIV strains to infect several CCR5‘CXCR4’ primary cells, including cells within immunoprivileged sites such as the brain (astrocytes and brain microvascular endothelial cells) and the testes (Leydig cells). This subset of HIV strains able to use an alternative coreceptor on primary cells not only includes HIV-1 subtype B, prevalent in the developed world, but also subtype C, the most globally prevalent HIV-1 subtype (Chapter 5). It is important that the identity of this coreceptor is determined, so the extent of its use by HIV and SIV, and its cellular distribution and expression levels, can be characterised, and its role in HIV pathogenesis of both subtype B and subtype C infections can be elucidated.
Such a coreceptor has implications with respect to both the targeting of new CD4$^+$ T-cell populations, as seen with expansion to use CXCR4 and infect naïve T-cells, but, more importantly, the advent of coreceptor-specific anti-HIV therapeutics. It is thus not unlikely to assume that when faced with a situation where the major coreceptor (CCR5) is removed, HIV subpopulations able to use this, or another, alternative coreceptor could thrive and become the dominant virus species present. Studies with other CCR5 inhibitors have shown that in the presence of CCR5 inhibitors, HIV can evolve to either use CXCR4, or a different region of CCR5 to avoid inhibition. It is therefore crucial that future work on these small molecule inhibitors of CCR5 developed at Pfizer GRD includes a thorough analysis of escape from inhibition. Information on the ease at which HIV could potentially escape from CCR5 inhibitors, and the mechanism by which this occurs, will be of vital importance to ensure the success of CCR5 inhibitors in HIV therapy. Nonetheless, although CCR5 inhibitors will undoubtedly contribute greatly to the control of the HIV epidemic, with over 42 million cases of HIV around the world, small virus populations with alternative coreceptor-using capabilities deserve to be closely watched.
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Human Leydig cells are productively infected by some HIV-2 and SIV strains but not by HIV-1

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**Objectives:** With the use of highly active antiretroviral therapy, the identification of HIV reservoirs within the body has become an important issue. However, the testis has been largely ignored despite representing a pharmacologic sanctuary which could act as a viral reservoir.

**Design:** Because alterations in testosterone production have frequently been reported in HIV-infected individuals, we investigated whether the testosterone-producing Leydig cells could become directly infected by HIV-1, HIV-2 or SIV.

**Methods:** Purified Leydig cells were infected with a panel of HIV-1, HIV-2 and SIV strains and examined for expression of HIV/SIV receptors. Additionally, the impact of CD4 transduction on Leydig cell infection was determined.

**Results:** Leydig cells were unable to support productive infection of the seven HIV-1 isolates tested. No CD4, CXCR4 or CCR5 expression was evident on the surface of Leydig cells and transduction with a CD4 expressing adenovirus did not induce HIV-1 infection. In contrast, some primary and laboratory adapted CD4-independent HIV-2 and SIV strains were able to enter and replicate productively in Leydig cells.

**Conclusions:** Our results suggest that Leydig cells do not represent a target for HIV-1 infection within the testis. In contrast, Leydig cells support HIV-2 and SIV infection in vitro and thus represent a potential target for infection in vivo. Receptor use and in vivo significance of HIV-2/SIV infection of Leydig cells remain to be determined.

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Keywords: Reservoir, male genital tract, Leydig cells, testis, HIV-1, HIV-2, SIV

**Introduction**

With the use of highly active antiretroviral therapy, the elucidation of HIV reservoirs within the body has become a major issue. The male genital tract could represent such a reservoir as numerous studies have...
demonstrated viral compartmentalization between blood and semen, suggesting a local production of viral particles (for a review see [1]).

The testis, like the brain, represents a pharmacologic sanctuary site due in part to the presence of membrane efflux receptors (P-glycoprotein) in the capillary endothelial cells of this tissue [2] which limit drug entry and in particular that of HIV protease inhibitors [3,4]. This organ has thus been hypothesized to represent a potential reservoir poorly accessible to antiretrovirals [5,6]. There are several endocrine and testicular dysfunctions observed in HIV positive men at different stages of the infection, such as changes in testosterone level and orchitis, which suggest that testes may become infected (for a review see [1]). Indeed, HIV-1 localization within the testis from asymptomatic seroconverters [7—11] has been obtained [7—13]. There are, to our knowledge, no data available concerning HIV-2 infection of the testis.

The testosterone-producing Leydig cells, within the testis, represent the first potential target cells for a blood-borne virus due to their localization next to the testicular blood vessels. Elevated levels of testosterone have been described in men during the early stages of the HIV infection, whereas hypogonadotropoemia was found in men with AIDS (for reviews see [1,14,15]). In some cases, these low serum testosterone levels were associated with high serum luteinizing hormone (LH) levels, implying a primary testicular failure [16]. We therefore investigated whether the testosterone-producing Leydig cells could become directly infected with HIV-1, HIV-2 and SIV isolates in vitro.

### Materials and methods

#### Viruses
All strains used in this study have been described previously (see Table 1). Virus titres were determined on CCR5 or CXCR4 NP2/CD4 cells.

#### Preparation and culture of human Leydig cells
The protocol was approved by local ethics committees. Normal testes were obtained from prostate cancer patients not subjected to any hormone therapy. To attest the functionality of the testes, spermatogenesis was verified by histological examination of a testis section. Isolation of Leydig cells was performed immediately following orchidectomy using a combination of mechanical dissociation, enzymatic digestion, filtration through nylon meshes and density Percoll gradient centrifugation [17]. Cells were left to adhere for 48 h on plastic dishes (Nunc, Roskilde, Denmark) in F-12/DMEM medium containing insulin (10 μg/ml), transferrin (10 μg/ml), vitamin C (0.1 mM), vitamin E (10 μg/ml), penicillin—streptomycin (50 U/l and 50 μg/ml, respectively), foetal calf serum (10%) and human chorionic gonadotropin (100 mU/ml; Organon, Puteaux, France). Following washes, purity of the Leydig cells was established to be > 98% using histochemical staining for 3β-hydroxysteroid dehydrogenase [18] and immunostaining for the LH receptor with a previously described antibody [19]. Contaminants were macrophages, peritubular cells and germ cells (<1% each).

#### Infectivity assays
One million Leydig cells were challenged for 16 h on day 3 of culture with 5 X 10⁴ focus forming units (FFU)/ml of each virus. Supernatants, sampled over 2 weeks, were assayed for reverse transcriptase (RT) activity by an ELISA (Lenti RT activity kit; Cavidi

### Table 1. Coreceptor use and replication of a panel of HIV-1, HIV-2 and SIV strains in isolated human Leydig cells.

<table>
<thead>
<tr>
<th>Strains tested</th>
<th>Type</th>
<th>Other receptors used</th>
<th>Replication</th>
<th>Infectivity titres</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SF162</td>
<td>R5, molecular clone [43]</td>
<td>R3, R8, STRL33</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>E80</td>
<td>R5, primary isolate [44]</td>
<td>R3</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>NLA.3</td>
<td>X4, TCLA [45]</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>2044</td>
<td>X4, primary isolate [46]</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>89.6</td>
<td>RSX4, molecular isolate [47]</td>
<td>R2b, R3, R8, CX3CR1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Gun wt</td>
<td>RSX4, TCLA [48]</td>
<td>R3, R8, GPR1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>2076</td>
<td>RSX4, primary isolate [46]</td>
<td>R3, R8, STRL33, GPR15</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>HIV-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ROD/B</td>
<td>X4, TCLA* [20]</td>
<td>—</td>
<td>—</td>
<td>1.7 X 10⁴</td>
</tr>
<tr>
<td>JAU</td>
<td>RSX4, primary isolate* [22]</td>
<td>R2b, R3, R8, APJ</td>
<td>*</td>
<td>8.7 X 10⁴</td>
</tr>
<tr>
<td>TER</td>
<td>R5, primary isolate* [22]</td>
<td>R2b, R3, R8, APJ, GPR1</td>
<td>+</td>
<td>6 X 10⁴</td>
</tr>
<tr>
<td>SAB</td>
<td>X4, primary isolate* [22]</td>
<td>R3</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>SIV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAC17 Efr</td>
<td>R5, molecular clone* [50]</td>
<td>R2b, R4, APJ, GPR1, STRL33, GPR15</td>
<td>+</td>
<td>2.7 X 10⁴</td>
</tr>
<tr>
<td>MAC17 Ecl</td>
<td>R5, molecular clone* [50]</td>
<td>R2b, R3, R8, GPR1, STRL33, GPR15</td>
<td>+</td>
<td>2.2 X 10⁴</td>
</tr>
</tbody>
</table>

*Coreceptors previously shown to mediate CD4-independent infection [25]. —, RT level < 5 pg/ml (same as control with no virus); +, peak RT level > 10 pg/ml and < 100 pg/ml except for *RT level reached 450 pg/ml in one culture but was below 20 pg/ml in two other cultures from different donors; ++, peak RT level > 800 pg/ml in three independent experiments. Cells were fixed at the end of the infection and immunostained for viral antigens to detect infected cells. Individual or clusters of stained cells counted as foci of infection and virus infectivity was estimated as FFU/ml. —, < 10 FFU/ml.
Leydig cell infection by HIV/SIV isolates Willey et al. 185

Following the final harvest of supernatant for RT analysis, Leydig cells were fixed and immunostained for viral antigen expression, as described previously for HIV-1 [20] and HIV-2/SIV [21]. Individual or clusters of stained cells counted as foci of infection and virus infectivity was estimated as FFU/ml.

**Determination of HIV/SIV receptor expression**

Flow cytometry experiments were carried out on $1 \times 10^6$ Leydig cells as described previously [22], using the anti-CXCR4 monoclonal antibody (MAB) 12G5 (2 $\mu$g/ml), anti-CD4 domain V1 MAb JAH7.3F11 (1 : 10) (both from the MRC AIDS reagent program), anti-CCR5 MAB 2D7 - phycoerythrin (PE) conjugate (20 $\mu$l) (BD Pharmingen, Heidelberg, Germany), anti-LH receptor MAB 29 (3 $\mu$g/ml) [19] or appropriate isotype controls at equivalent concentrations.

Reverse transcription–PCR was performed as described previously [23]. Primers used for reverse transcription–PCR were either described previously [24–30] or designed according to the Primer 3 software [31]. The amplification products were validated by direct sequencing using an automated DNA sequencer (373 DNA sequencer, Applied Biosystems, Foster city, California, USA).

**Adenovirus-CD4 enhancement assay of HIV and SIV infection in Leydig cells and CD4-negative cell lines**

Cells (1.5 $\times 10^5$) were incubated overnight at 37°C with 200 µl of medium (controls) or with a previously described replication-defective adenovirus expressing the human CD4 gene (Adenovirus-CD4) [32], at a dosage giving maximum infection of NP2/CCR5 cells with an R5-tropic CD4-dependent HIV-1 isolate, as determined by titration. The adenovirus or medium was then washed off and 200 µl of virus stock added for 16 h. At 12 days post-infection, cells were fixed and stained for viral antigens [20,21] and infectivity titres were calculated in FFU/ml.

**Results**

**Susceptibility of isolated human Leydig cells to infection by a panel of HIV-1, HIV-2 and SIV strains**

In three independent cell cultures derived from different donors, no replication was observed for any of the seven HIV-1 isolates tested, whether they were R5, X4 or dual-tropic strains (Table 1). Similarly, immunostaining for the p24 protein revealed no HIV-1 infected cells (Table 1). In contrast, the HIV-2 strain ROD/B replicated efficiently in three independent Leydig cell cultures (Table 1) with RT activity peak consistently $> 800$ pg/ml. A low level of productive infection of Leydig cells (RT $< 100$ pg/ml) was also consistently observed for the primary HIV-2 isolates JAU and TER, whereas no replication was observed for the primary isolate SAB. These results were correlated by the immunodetection of viral protein in cell cultures infected by ROD/B, JAU and TER 2 weeks post-infection, while no staining was observed for SAB (Table 1). Leydig cell infection was also observed for two SIV strains, SIVsmB670 and SIVmac17Efr, while no RT activity or viral protein production was evident following SIVmac17Ecl challenge (Table 1).

**HIV/SIV receptor and coreceptor expression on human Leydig cells**

Flow cytometric analysis showed that Leydig cells lacked CD4, CXCR4 and CCR5, while they stained positive for the LH receptor, a specific Leydig cell marker [33] (Fig. 1). Using reverse transcription–PCR, no mRNA encoding CCR5, CCR1, CCR2b, CCR3, CCR8, CX3CR1, APJ, GPR15, STRL33 or ChemR23 were detected in three independent cultures (data not shown). A weak expression of CD4, CXCR4 and
GPR1 mRNAs was detected in the three cultures tested at 35 cycles of PCR (data not shown). This expression correlated with the detection of a faint band corresponding to a CD14 amplification product, confirming a very low contamination of our cultures with macrophages (<1%).

**CD4 enhancement assay**

Leydig cells were transfected with a cDNA encoding the human CD4 receptor using a replication-defective adenovirus vector (CD4-Adenovirus) [32] and were subsequently infected with a panel of HIV-1, HIV-2 and SIV isolates (Fig. 2). At 12 days post-infection, cells were fixed and immunostained for viral proteins. No infection was detected for the three HIV-1 strains tested, or SIVmac17Ecl, either with or without CD4-Adenovirus. In contrast, in the presence of CD4, the infectivity titre of the HIV-2 isolates JAU and TER dramatically increased by 14- and 50-fold, respectively (Fig. 2). HIV-2 ROD/B infectivity also increased, although to a much lower extent (about threefold). No significant differences were observed for SIV strains.

**Discussion**

The alterations in circulating testosterone concentrations observed in asymptomatic HIV-positive individuals and in AIDS patients (reviewed in [1,14,15]) prompted us to investigate if testosterone producing human Leydig cells were susceptible to HIV and SIV infection. Indeed, other human cells of similar mesenchymal origin have been reported to be productively infected by HIV in vitro [34–36].

No productive infection was ever detected for the seven HIV-1 isolates tested, regardless of coreceptor specificity or CD4 transduction of Leydig cells. Using flow cytometry, we were unable to detect CD4, CXCR4 or CCR5 expression on the surface of isolated Leydig cells, which confirmed previous immunohistochemistry results on human testicular sections [37]. A very weak expression of CD4, CXCR4 and GPR1 mRNA was observed using reverse transcription–PCR, most probably due to the slight contamination of the Leydig cell cultures by macrophages (<1%), as CD14 mRNA was also detected. CCR5 mRNA was not detected, nor was mRNA encoding 10 previously described HIV/SIV alternative 7TM receptors [38,39]. The absence of HIV-1 coreceptors on the cell surface was further confirmed by the fact that CD4 expression by Leydig cells did not induce HIV-1 infection. Although we cannot completely rule out that Leydig cells can be latently infected, our results strongly suggest that Leydig cells do not represent a significant target of HIV-1 in vivo. The alterations in testosterone production observed in HIV-1 infected individuals are therefore unlikely to result from a disruption of Leydig cell metabolism due to HIV-1 replication cycle. Whether interactions between the viral envelope and the plasma membrane could account for this phenomenon remains to be determined.

It has been shown previously that HIV-2 and SIV strains more readily infect CD4− cells and exhibit expanded coreceptor tropism in vitro compared with HIV-1 strains (for reviews see [39,40]). Here, productive infection of Leydig cells was observed for three CD4-independent HIV-2 strains and two CD4-independent SIV strains that are multi-coreceptor tropic on CD4-positive cells (Table 1) [22]. In vitro, CD4-independent entry of HIV-2 and SIV strains through 7TM receptors other than CXCR4 and CCR5 has been reported (for a review see [39]). Some HIV-2 and SIV strains can also infect peripheral blood mononuclear cells independently of either CCR5 or CXCR4 [38], implicating a potential role of at least some alternative receptor(s) for infection in vivo. The receptor specificity of HIV-2/SIV entry into Leydig cells is presently not known, as we failed to unequivocally detect an already characterized receptor. However, HIV-2 ROD/B infection of Leydig cells could be neutralized by sera from HIV-2-positive individuals and by the ROD/B envelope specific MAb28.8e [41], indicating that entry into Leydig cells is via specific viral envelope–cell membrane interactions (data not
shown). CD4 transduction of Leydig cells enhanced infectivity of HIV-2 strains ROD/B, JAU and TER which suggest that CD4 binding either allowed more efficient utilization of a previously used receptor or induced use of another coreceptor. Indeed, the use of CD4 usually enables a wider range of receptors to be exploited for infection [39]. In contrast, CD4 transduction could not induce infection by X4 only using HIV-2 SAB, further demonstrating that CXCR4 is not expressed by these cells.

The viral compartmentalization observed for the male genital tract suggests that strain selection can occur in vivo. Primary HIV-2 and SIV strains can exhibit broad in vitro tropism and varying degrees of CD4-independence [20,42], thus Leydig cell tropic HIV-2 and SIV strains could be selected for in the testicular immunoprivileged site. The in vivo significance of our findings that Leydig cells are susceptible to HIV-2 and SIV infection remain to be determined and we believe that the testicular status of HIV-2 infected individuals and SIV-infected monkeys deserves more attention.

Acknowledgements

The authors are very grateful to the surgeons who made this study possible: Dr Menut (Polyclinique du Trégor, Lannion), Dr Tiengou (CHU R, Vannes) and Dr Triffard (Clinique Ste Thérèse, Saint Brieuc). The drug transporter P-glycoprotein limits oral absorption and brain entry of HIV-1 protease inhibitors. *J Clin Invest* 1998, 101:289–294.


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Identification of a Subset of Human Immunodeficiency Virus Type 1 (HIV-1), HIV-2, and Simian Immunodeficiency Virus Strains Able To Exploit an Alternative Coreceptor on Untransformed Human Brain and Lymphoid Cells

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Received 3 December 2002/Accepted 4 March 2003

The chemokine receptors CCR5 and CXCR4 are the major coreceptors for human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV). At least 12 other chemokine receptors or close relatives support infection by particular HIV and SIV strains on CD4+ transformed indicator cell lines in vitro. However, the role of these alternative coreceptors in vivo is presently thought to be insignificant. Infection of cell lines expressing high levels of recombinant CD4 and coreceptors thus does not provide a true indication of coreceptor use in vivo. We therefore tested primary untransformed cell cultures that lack CCR5 and CXCR4, including astrocytes and brain microvascular endothelial cells (BMVECs), for naturally expressed alternative coreceptors functional for HIV and SIV infection. An adeno-virus vector (Ad-CD4) was used to express CD4 in CD4+ astrocytes and thus confer efficient infection if a functional coreceptor is present. Using a large panel of viruses with well-defined coreceptor usage, we identified a subset of HIV and SIV strains able to infect two astrocyte cultures derived from adult brain tissue. Astrocyte infection was partially inhibited by several chemokines, indicating a role for the chemokine receptor family in the observed infection. BMVECs were weakly positive for CD4 but negative for CCR5 and CXCR4 and were susceptible to infection by the same subset of isolates that infected astrocytes. BMVEC infection was efficiently inhibited by the chemokine vMIP-I, implicating one of its receptors as an alternative coreceptor for HIV and SIV infection. Furthermore, we tested whether the HIV type 1 and type 2 strains identified were able to infect peripheral blood mononuclear cells (PBMCs) via an alternative coreceptor. Several strains replicated in CCR5 homozygous for a 32-bp deletion in CCR5 (23, 47, 58), but none replicated in CCR5 homozygous for a 32-bp deletion in CCR5 (23, 47, 58). CXCR4-using variants emerge late in disease in up to 50% of AIDS patients (72). This change in coreceptor use correlates with disease progression in infected individuals (19, 60), although it is not a prerequisite, as not all infected individuals demonstrate a coreceptor switch (26). While primary X4 strains can infect macrophages via CXCR4 (67, 75, 77), these variants primarily target new populations of T cells that express CCR5 but not CCR5, e.g., naïve T-cells (7, 52).

Although CCR5 and CXCR4 are the major coreceptors used in vivo, there are at least 12 other members of the chemokine receptor family, and related "orphan" receptors, that can support infection of indicator cell lines in vitro (5, 16, 55). These include CCR3 (14, 28), CCR8 (53), GPR1 (34, 63), GPR15 (34), CXCR5 (3, 25), Apj (13, 31), and RDC1 (64). In general, HIV type 2 (HIV-2) and SIV strains use a wider range of these alternative coreceptors than HIV-1, frequently as efficiently as they use CCR5 and/or CXCR4 (16, 53). For HIV-1,
there is little current evidence to indicate that alternative coreceptors (other than CCR5 and CXCR4) contribute to viral replication in vivo (79). The ability of HIV-1 strains to exploit alternative coreceptors on the surfaces of cell lines therefore does not provide a true indication of coreceptor usage properties in vivo. The capacity of naturally expressed coreceptors (other than CCR5 and CXCR4) to support HIV infection of primary cell cultures may provide a stronger indication for their use in vivo. Thus, it has been reported that a maternal isolate used CXCR6 in addition to CCR5 and CXCR4 on indicator cell lines and replicated in CCR5- peripheral blood mononuclear cells (PBMCs) in the presence of a CXCR4 inhibitor (61, 79). Similarly, Lee et al. reported that CCR8 supported infection of primary thymocytes by particular HIV-1 isolates (45).

In this study, we have investigated whether functional coreceptors can be detected on different primary or untransformed human cell cultures. We report the identification of a subset of HIV and SIV strains that are able to exploit an unknown coreceptor naturally expressed on brain microvascular endothelial cells (BMVECs) and astrocyte cultures. Moreover several of these HIV-1 and HIV-2 strains were able to replicate in CCR5- PBMCs with CXCR4 blocked by the bicyclam AMD3100. Replication of such strains was inhibited by the chemokine vMIP-I, implicating an unknown vMIP-I receptor as the coreceptor involved.

**MATERIALS AND METHODS**

**Viruses.** SF162 is a non-syncytium-inducing molecular clone of HIV-1 which uses primarily CCR5 as a coreceptor (12). The HIV-1 isolate 2044 utilizes CXCR4 as a coreceptor and has been previously described (68). GUN-1v is a variant of the T-cell-line-adapted HIV-1 isolate GUN-1vcp, which was isolated by its ability to infect brain-derived BT cells derived from human meningioma tissue (77). The HIV-1 strain 89.6 is an R5X4 molecular clone of HIV-1 (18), as is the isolate HAN-2 (59). The R5X4 HIV-1 isolate P1019 is a pediatric strain isolated from a 3-year-old child (36). All isolates of HIV-1 used in this study are clade B. The HIV-2 isolates used here are all Portuguese primary isolates from individuals of West African descent (50, 54). ALI was derived from a patient with AIDS-related complex. TER, JAU, MIL, and SAB were isolated from AIDS patients, and the R5X4 isolates JAU, MIL, and ETP originated from symptomatic patients (54). The SIV strain SIVmac17Efr is a molecular clone of the isolate SIVmac239, containing the env and nef genes and the entire 3' long terminal repeat from the neuroviral SIVmac239 clone SIV17E-Br (35). SIVmac4 is a T-cell-line-adapted variant of SIVmac239 (51). The coreceptor use of most of the virus isolates used in this study has been previously described (46, 54) and is summarized in Table 1. All virus stocks were propagated in PBMCs purified from whole blood by density gradient centrifugation and stimulated for 2 days in phytohemagglutinin (1 μg/ml) (Sigma) and for 2 days in human recombinant IL-2 (20 U/ml) (Roche, Inc.), with the exception of GUN-1v, which was passaged in the T-cell line MOLT4 clone 8. The replication-defective adenovirus vector carrying the human CD4 gene (Ad-CD4) (76) was propagated in the cell line 293. When cytopathology was observed, cells were pelleted by centrifugation, the supernatant was discarded, and the cells were resuspended in fresh Dulbecco's modified Eagle's medium (DMEM) (Gibco Invergent Corporation) with 10% fetal bovine serum (FBS). Cells were lysed by freeze-thawing three times to release intracellular virus, and cell debris was removed by centrifugation. Ad-CD4 was titrated on NP2/CCR5 cells (see below) and subsequently challenged with serially diluted SF162. The Ad-CD4 dilution that conferred maximum SF162 infection was used in all further experiments.

**Cell lines.** The human glioma-derived cell lines U87/CD4, stably expressing the chemokine receptor CCR2b (6, 25), and NP2/CCR5, stably expressing CCR3, CCR5, CCR8, and GPR1 (69), were cultured in DMEM supplemented with 10% FBS, gentamicin (10 μg/ml) (Gibco Invergent Corporation), and puromycin (1 μg/ml). NP2/CD4/Apj cells were made by transfecting parental NP2/CD4 cells with a pBabe (puro)-Apj construct and selecting for stable transfectants in puromycin-containing selection medium. The T-cell line MOLT4 clone 8 was obtained from the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program (22, 41) and were maintained in RPMI 1640 medium (Gibco Invergent Corporation) containing gentamicin (10 μg/ml) (Gibco Invergent Corporation) and 10% FBS.

**Primary cells.** PBMCs from an individual homozygous for wild-type (wt) CCR5 or for the 32-bp deletion were prepared from whole blood, drawn under procedures approved by the University of Massachusetts Medical School Institutional Review Board, and stimulated with phytohemagglutinin and interleukin-2 as described above. Primary BMVECs (Clonetics Inc.) were maintained in endothelial cell basal medium 2 supplemented with EGM-2 additives (Clonetics Inc.). Fetal astrocytes were prepared from primary human fetal brain tissue, while human adult astrocytes were from temporal lobectomy samples. The use of fetal brain samples was approved by the University of Massachusetts Institutional Review Board (no. 10353). Following dissection and removal of blood vessels,
AOP-RANTES was provided by Amanda Proudfoot, Serono Inc, Geneva, Switzerland. The chemokines 1309 and eotaxin and the human herpesvirus 8 (HHV8)-encoded chemokines vMIP-I and vMIP-II were purchased from R&D Systems, Inc. The CXCR4 antagonist AMD3100 was provided by AnonMed Inc, Langhorne, Pennsylvania. The CXCR4 inhibitor TAK-779 was obtained through the AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, NIH (4). The glioblastoma fibrillary acidic protein (GFAP) monoclonal antibody (MAb) used was a rabbit anti-cow GFAP from Dako. The CD45 MAb 2D7 was from the NIH AIDS Research and Reference Reagent Program. The CD4 MAb 12G5 was provided through the NIH AIDS Research and Reference Reagent Program from James Hoxie (32). The CD4 antibody 6140 WILLEY ET AL. J. ViroL is directed to the N-terminal domain of CD4 and was provided through the NIH AIDS Research and Reference Reagent Program. The CXCR4 MAb 12G5 was provided through the NIH AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, NIH (4). The N-terminally modified RANTES analogue 6140 WILLEY ET AL. J. ViroL efficiencies, whereas recovery from dispersed temporal lobectomy samples was markedly reduced.

Inhibitors and antibodies. The chemokine RANTES was purchased from PeproTech Inc., Rocky Hill, N.J. The N-terminally modified RANTES analogue AOP-RANTES was provided by Amanda Proudfoot, Serono Inc, Geneva, Switzerland. The chemokines 1309 and eotaxin and the human herpesvirus 8 (HHV8)-encoded chemokines vMIP-I and vMIP-II were purchased from R&D Systems, Inc. The CXCR4 antagonist AMD3100 was provided by AnonMed Inc, Langhorne, Pennsylvania. The CXCR4 inhibitor TAK-779 was obtained through the AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, NIH (4). The glioblastoma fibrillary acidic protein (GFAP) monoclonal antibody (MAb) used was a rabbit anti-cow GFAP from Dako. The CD45 MAb 2D7 was from the NIH AIDS Research and Reference Reagent Program. The CD4 MAb 12G5 was provided through the NIH AIDS Research and Reference Reagent Program from James Hoxie (32). The CD4 MAb 5A8, directed to the D2 domain of CD4, has been described previously (9). Q4120 is directed to the N-terminal domain of CD4 and was provided through the Central facility for AIDS Research from Quentin Stottard (30).

Immunostaining for fluorescence microscopy. Glass coverslips (13-mm diameter) were placed in the wells of a 24-well plate, sterilized by washing in 70% ethanol, and rinsed in sterile phosphate-buffered saline (PBS). Cells were plated at the appropriate density (primary astrocytes at 1 x 10^5/ml and cell lines at 4 x 10^5/ml) and left overnight at 37°C to adhere. Cells for GFAP staining were washed once in PBS before fixation in a cold (−40°C) 1:1 methanol-acetone mix for 5 to 10 min. Immunostaining for cell surface proteins was carried out at 4°C before fixation in a cold (−40°C) 1:1 methanol-acetone mix. Cells were gently rinsed in PBS before incubation with the appropriate antibody diluted to 5 μg/ml in PBS−1% FBS. Antigen was detected with a goat anti-mouse fluorescein isothiocyanate conjugate (Dako) diluted 1:40 or a swine F(ab')2 anti-rabbit fluorescein isothiocyanate conjugate (Dako) diluted 1:15 in PBS−1% FBS. Images were captured on a confocal microscope and analyzed with the Confocal Assistant software.

Infectivity assays. Cells were plated the day before use in a 48-well tray at 3 x 10^5 cells/ml in 500 μl of the appropriate medium. On the day of infection, medium was removed and cells were incubated with 100 μl of 10-fold serially diluted cell-free virus. After incubation for 3 h, cells were gently washed twice with growth medium and incubated for 72 h in 500 μl of fresh medium. For adenovirus expression of CD4, cells were plated at 3 x 10^4 cells/ml in 500 μl of the appropriate medium. The following day, medium was removed and cells were exposed to 100 μl of Ad-CD4 for 3 h. The amount of Ad-CD4 added corresponded to the dose of virus that conferred maximum SF162 infectivity to NP2/CCR5 cells. Cells were then rinsed and left overnight at 37°C. The next day, medium was removed and cells were exposed to HIV as described above. Infected cells were detected by immunostaining for intracellular p24 as described below.

Infection assays. Adherent astrocytes and BMVECs were seeded the day before infection in 48-well plates at 3 x 10^5 cells/ml in 500 μl of the appropriate medium. On the day of infection, medium was removed and cells were incubated with 1 h for 75 μl of inhibitor (i.e., chemokines, AMD3100, or antibodies) at a 2X final concentration before being exposed to approximately 100 focus-forming units (FFU) of virus in 75 μl for 3 h. Cells were washed once in medium before the inhibitor was replaced at a 1X concentration (chemokines and small molecule compounds at 500 nM and Mabs at 10 μg/ml) and cells were left at 37°C for 72 h. PBMCs were seeded in 100 μl in V-bottom 96-well plates at 10^5 cells/ml before centrifugation for 5 min at 210 x g and resuspension in 50 μl of medium alone or with inhibitor. Following incubation for 1 h at 37°C, 50 μl of virus at 10^3 FFU/ml or higher was added and mixed, and cells were incubated at 37°C for 3 h. After incubation, cells were washed three times by centrifugation for 5 min at 210 x g in medium and resuspended in 150 μl of 1X inhibitor. Cells were left at 37°C, and cell-free supernatant was harvested on days 0, 3, 6, 9, 12, 15, and 18 of infection to monitor virus production. The inhibitor was replenished at each harvest. Inhibition assays that tested the effect of mouse Mabs, e.g., the anti-CCR5 2D7, and anti-CD4 Q4120, were directly compared with those for isotype antibody controls (immunoglobulin G1 [IgG1]). These antibodies were used at 5 μg/ml in these inhibition assays. Thin high concentration of isotype control nonspecifically reduced the infectivity of tested viruses by up to 50%.

Measurement of virus infectivity. HIV infection of PBMCs was determined by measuring reverse transcriptase (RT) activity in cell supernatants by an RT enzyme-linked immunosorbent assay (CavidiTech, Uppsala, Sweden). Infected adherent cells were detected by immunostaining for intracellular p24 as previously described (17, 49). In brief, cells were rinsed in PBS before being fixed in a cold (−40°C) 1:1 methanol-acetone mix for 5 to 10 min and rinsed once in PBS and once in PBS−1% FBS. For HIV-1, cells were stained with a 1:1 mix of anti-HIV-1 Gag Mabs 38:98R and EF7 (Medical Research Council [MRC] AIDS Reagent Program, Potters Bar, England) diluted 1:40, while HIV-2-infected cells were stained with a mix of six HIV-2-positive serum samples (WHO panel C; MRC AIDS Reagent Program) diluted 1:4,000. Infected cells were detected with a 1:400 dilution of a goat anti-mouse or goat anti-human β-galactosidase conjugate for HIV-1 and HIV-2, respectively (Southern Biotechnology Associates, Inc.) and revealed with an X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) substrate (PBS with 3 mM potassium ferrocyanide, 3 mM potassium ferrocyanide, 1 mM magnesium chloride, and 0.5 mg of X-Gal per ml).

RT-PCR. Total cellular RNA was extracted from 1 x 10^6 to 5 x 10^6 cells by using the RNaseasy kit from Qiagen, and mRNA was isolated by using the Oligotex kit from Qiagen. Specific mRNA sequences were amplified with the primers described in Table 2, using the Titanium one-step RT-PCR kit (Clontech). The housekeeping glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was amplified from all cell samples as a positive control, and negative controls were primers for a gene known not be expressed in that cell type. All reactions were carried out in duplicate, with control reactions taking place on untranscribed mRNA to demonstrate the absence of contaminating genomic DNA. All reaction products were run on a 1.4% agarose gel with a 100-bp DNA ladder (New England Biolabs).

RESULTS

Lack of CCR5 and CD4 on cultured astrocytes by immunostaining. Untransformed cultured fetal astrocytes (F01) and cells derived from adult temporal lobectomies (samples 001A and 004.2) were tested for their expression of the astrocyte marker GFAP. Figure 1 shows that fetal astrocytes were strongly positive, while both adult cultures showed weaker and more diffuse staining, consistent with the observations of Marriott et al. (48). We also tested for CCR5, CXCR4, and CD4 expression on each of the untransformed astrocyte cultures and found that purified fetal astrocytes (F01) cultured for 2 weeks expressed neither CCR5 nor CD4 but were weakly positive for CXCR4, as shown previously (Fig. 1) (53). CD4, CCR5, and CXCR4 were not detected on either of the adult brain-derived cultures 001A and 004.2.

CD4 expression on cultured astrocytes confers sensitivity to a subset of HIV and SIV strains. Astrocytes do not usually express CD4 and therefore support only an inefficient infection by particular HIV-1 strains (56, 74). The chemokine receptor expression profile of astrocytes is controversial, and the role of coreceptors in the observed low-level infection is unclear (5, 56). In order to determine if the cultured fetal and adult GFAP-positive cultures express functional coreceptors, we used an Ad-CD4 vector to express CD4 on these cells. As seen in Fig. 2A, infection of the cell line NP2/CCR5 with Ad-CD4 is sufficient to confer infection by R5 viruses to levels comparable to those seen with NP2 cells stably expressing both CD4 and CCR5. By the same principle, infection of astrocyte cultures with Ad-CD4 enables the screening of a range of HIV and SIV isolates for their capacity to use functional coreceptors expressed on astrocytes that lack CCR5 (fetal) or both CCR5 and CXCR4 (adult). Adult astrocyte cultures 001A and
TABLE 2. Oligonucleotide sequences used for the amplification of HIV and SIV receptors and coreceptors

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'→3')</th>
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<tr>
<td>GAPDH</td>
<td>TGGATATTGCCATCAATGACC GATGCCATGGACTGTGGTCATG</td>
</tr>
<tr>
<td>CD4</td>
<td>TCAGGGAAAAGAAAGTGGTGCA AAGAAGGAGCCCTGATTTCC</td>
</tr>
<tr>
<td>CCR5</td>
<td>CTTCAATTACACCTGACGTCCT CACAGCCCTGGCCCTTTTCT</td>
</tr>
<tr>
<td>CXCR4</td>
<td>TAGATATCATCCATGGAGGGGATCAG TAGCGGCGCCAAGCTGGAGTGAAAATGGCAG</td>
</tr>
<tr>
<td>CCR8</td>
<td>GCAAGTTGCTCCTGGCATGTC CATGGGTGGCATAAGTCAGC</td>
</tr>
<tr>
<td>CCR3</td>
<td>GTGTGATCCAGAGCACTGATG CAACAAAGGCGTAGATCACCG</td>
</tr>
<tr>
<td>GPR1</td>
<td>GCTGATACGAGAGCAGGTATG CAAACAAAGGCTGAGATCCACCCG</td>
</tr>
<tr>
<td>GPR15</td>
<td>ATGGACCCAGAAGAAGCCTC TTAGAGTGAACAGACACCCTC</td>
</tr>
<tr>
<td>CXCR6</td>
<td>CAGGCATGGCGGAGGATCAG TAGCGGCGCCAAGCTGGAGTGAAAATGGCAG</td>
</tr>
<tr>
<td>RDCl</td>
<td>AAAGAGATGTTACGCGCCTGACGTCATGGAGGGGATCAG</td>
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004.2) preinfected with Ad-CD4 were resistant to HIV-1 strains that predominantly used CCR5 (SF162) or CXCR4 (2044). In contrast, three R5X4 HIV-1 strains (P1019, HAN-2, and GUN-1v) and several HIV-2 and SIV strains (TER, ETP, SIVmac17Efr, and SIVmac17Efr) were able to infect adult astrocytes 001A and, to various extents, 004.2 (Fig. 2B and C). The fetal astrocyte culture F01 weakly expressed CXCR4 (Fig. 1), and adenovirus-mediated expression of CD4 conferred sensitivity to all isolates that use CXCR4, including the HIV-1 X4 isolate 2044 (Fig. 2D). However, it is likely that the HIV-2 strain TER and SIVman4 exploit an alternative coreceptor for infection of fetal astrocytes, since TER does not use CXCR4 on standard CD4+ indicator cell lines (54), and CXCR4 use by SIVmac is inefficient and at least 1,000 times less than CCR5 use (data not shown).

Infection of fetal and adult astrocytes by HIV-1, HIV-2, and SIV strains in the absence of CD4 expression. Low-level CD4-independent infection of brain cultures has been reported for various HIV and SIV strains (15,56). Therefore, we also tested whether fetal and adult astrocytes supported infection by the same panel of HIV and SIV strains that infected astrocytes 001A and 004.2 (Fig. 2B and C). The fetal astrocyte culture F01 weakly expressed CXCR4 (Fig. 1), and adenovirus-mediated expression of CD4 conferred sensitivity to all isolates that use CXCR4, including the HIV-1 X4 isolate 2044 (Fig. 2D). However, it is likely that the HIV-2 strain TER and SIVman4 exploit an alternative coreceptor for infection of fetal astrocytes, since TER does not use CXCR4 on standard CD4+ indicator cell lines (54), and CXCR4 use by SIVman4 is inefficient and at least 1,000 times less than CCR5 use (data not shown).

Infection of fetal and adult astrocytes by HIV-1, HIV-2, and SIV strains in the absence of CD4 expression. Low-level CD4-independent infection of brain cultures has been reported for various HIV and SIV strains (15,56). Therefore, we also tested whether fetal and adult astrocytes supported infection by the same panel of HIV and SIV isolates in the absence of adenovirus-mediated CD4 expression. These cultures were negative for CD4 expression by immunohistochemical methods, and infection by most strains was weak, as expected (Fig. 3). The HIV-1 strains SF162 (R5) and 2044 (X4) did not infect either the adult or fetal astrocytes. The R5X4 isolates HAN-2 and P1019, as well as the HIV-2 strain TER and the SIV isolates Man4 and 17Efr, gave variable but low infectivity titers on all three astrocyte cultures. The HIV-1 isolate GUN-1v was able to infect both adult astrocyte cultures 004.2 and 001A, even though infection of coreceptor indicator cell lines in the absence of CD4 was negative (data not shown).

Infection of fetal and adult astrocytes is reduced by chemokine receptor ligands. In order to identify the possible coreceptor(s) being exploited for infection, we tested a range of chemokine receptor ligands for their capacity to block infection by HIV-1 GUN-1v. A common feature of the HIV and SIV strains that infected astrocytes was their capacity to exploit CCR3, CCR8, and GPR1 as coreceptors on indicator cell lines (in addition to CCR5 and sometimes CXCR4). We therefore included ligands that bind these chemokine receptors in the inhibition assays. The activities of all coreceptor-specific chemokines and antibodies were determined by carrying out control inhibitions on appropriate coreceptor-expressing cell lines. CCR3- and CCR8-mediated HIV infection was selectively inhibited by the ligands eotaxin and IL8, respectively (data not shown). In comparison, vMIP-I (an HHV-8-encoded chemokine) inhibited infection of control cell lines expressing CCR3, GPR1, and CXCR6, as previously reported (66), and weakly blocked CCR5-mediated infection (data not shown).

GUN-1v infection of adult astrocytes 001A pretreated with Ad-CD4 was reduced to approximately 50% of the levels seen in the absence of inhibitors by both vMIP-I (which binds CCR8, GPR1, and CXCR6) and RANTES (which binds CCR5, CCR1, CCR3, and CCR9). The ligand eotaxin (CCR3, D6) also reduced infectivity to approximately 60%. (Fig. 4A). TAK-779, the CCR5 small-molecule inhibitor, had no effect on GUN-1v infection despite inhibiting CCR5-expressing indicator cell lines by 100% (data not shown). In addition, the CCR5-specific MAb 2D7 failed to block astrocyte
FIG. 1. Immunostaining of astrocyte cultures. All astrocyte cultures and control cells were seeded onto 13-mm-diameter glass coverslips the day before being stained for CD4 (Q4120), CCR5 (2D7), CXCR4 (12G5), and GFAP, a marker for astrocytes. Nuclei of antigen-negative cells were stained with propidium iodide (red).
FIG. 2. CD4 expression on astrocytes confers susceptibility to infection with HIV and SIV. (A) The activity of Ad-CD4 was tested by pretreating NP2/CCR5 cells with Ad-CD4 for 3 h, rinsing, and incubating overnight. Ad-CD4-pretreated and untreated NP2/CCR5 cells, as well as NP2 cells stably expressing CD4 and CCR5, were exposed to the R5-using HIV-1 strain SF162 for 3 h, and infected cells detected by p24 immunostaining were counted after 72 h. (B) Untransformed GFAP-positive adult astrocytes 001A and 004.2 were pretreated with Ad-CD4 before infection with the HIV-1, HIV-2, and SIV strains shown. After 72 h, cells were fixed and stained for intracellular viral antigens. (C and D) The titer of each HIV or SIV isolate was assessed on adult astrocytes 001A and 004.2 (C) and fetal astrocytes F01 (D). Cells were exposed to Ad-CD4 and infected as described above. All HIV and SIV isolates tested had titers of between $1 \times 10^5$ and $5 \times 10^5$ FFU/ml on NP2/CD4 coreceptor-expressing indicator cells (Table 1). Infected cells were detected by immunostaining for viral antigens and counted. All data are representative of those from at least three independent experiments.

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infection in comparison to an IgG1 isotype control. Taken together, these data confirm that the inhibition observed by RANTES is not due to the expression and use of CCR5 on these cells. Astrocyte infection was not affected by the CXCR4 antagonist AMD3100 or the CCR5-specific ligand I309. The same chemokines that weakly reduced infection of CD4⁺ adult astrocytes 001A (vMIP-I, eotaxin, and RANTES) also inhibited infection in the absence of CD4 by between 50 and 60% (Fig. 4B). Intriguingly, infection of CD4⁺ 001A cells was completely blocked by the CD4 MAb Q4120, indicating the presence of low levels of CD4 undetectable by immunostaining (Fig. 1) but sufficient to explain the observed infection by the HIV-1 strains GUN-1v and HAN-2, in the absence of adenovirus-mediated CD4 expression. As seen in Fig. 4A, CD4⁺ astrocyte infection was also unaffected by 2D7. Infection of the fetal astrocyte preparation F01 by GUN-1v was reduced to less than 10% of control levels by AMD3100 and was minimally affected by RANTES, I309, and vMIP-I, confirming that CXCR4 conferred the majority of infection in these cells (Fig. 4C). The observation that adult astrocyte infection can be inhibited by chemokines implies a role for a chemokine receptor(s) in virus entry into these cells. However, weak inhibition was observed with several chemokines, all of which interact with a range of chemokine receptors (vMIP-I, RANTES, and eotaxin), suggesting that the chemokine receptor exploited for infection of astrocytes is not a major target for these chemokines.

BMVECs support infection by the same viruses shown to infect astrocytes via an unidentified coreceptor. We next tested the ability of primary BMVECs to support infection by a range of HIV-1, HIV-2, and SIV isolates. No infection by HIV-1 and HIV-2 isolates that predominantly use CCR5 or CXCR4 was observed. However, the same subset of viruses that infected adult astrocytes also replicated in BMVECs. These included R5X4 HIV-1 strains (GUN-1v and HAN-2), HIV-2 strains (TER), and SIV strains (17Efr) (Fig. 5A). In addition, the R5X4 HTV-2 isolates JAU and MLC and the SIV variant SIVmac316 infected BMVECs. BMVEC infection by GUN-1v was completely inhibited by vMIP-I (Fig. 5B) but was unaffected by ligands for CCR5 (AOP-RANTES), CXCR4 (AMD3100), and CCR8 (I309), despite their ability to block infection of coreceptor-expressing cell lines (data not shown). The CCR3 ligand eotaxin reduced BMVEC infection by approximately 30%; however, the chemokine analogue vMIP-II, a more potent inhibitor of CCR3, had no effect on infection (43, 66). BMVEC infection was reduced to less than 5% by the CD4 MAb Q4120 (D1) and 5A8 (D2/3), indicating that CD4 was present on the cell surface at levels high enough to support

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**FIG. 3.** Infection of fetal and adult astrocytes by HIV-1, HIV-2, and SIV without Ad-CD4 pretreatment. Adult 004.2 and 001A astrocytes (A) and fetal F01 astrocytes (B) were seeded the day before infection with a panel of HIV and SIV isolates for 3 h. Infected cells were detected by immunostaining for intracellular viral p24 after 72 h. Titers are representative of those from three independent experiments. All viruses tested had infectivity titers of between 1 x 10⁵ and 5 x 10⁶ FFU/ml on NP2/CD4 coreceptor-expressing indicator cells.
FIG. 4. Infection of CD4+ astrocytes is partially inhibited by vMIP-I and RANTES. Adult-derived astrocytes 001A either pretreated with Ad-CD4 (A) or untreated (B) and fetal astrocytes F01 (C) were preincubated with a range of chemokines or small-molecule inhibitors at 500 nM or with antibodies at 5 μg/ml before being exposed to the HIV-1 isolate GUN-lv. Infected cells were detected by immunostaining for p24 at 72 h postinfection. One hundred percent infection represents the titer in the absence of inhibitor and was approximately 150 FFU. All values were calculated with respect to this control. All samples were done in duplicate, and the error bars represent the standard errors of the means.
FIG. 5. BMVECs support infection by the same subset of HIV and SIV isolates that infect astrocytes. (A) Untransformed BMVECs were exposed to HIV and SIV strains, and intracellular viral antigens were detected by immunostaining following a 72-h incubation. (B) The sensitivity of BMVEC infection to chemokine inhibition was determined by preincubating cells with chemokines or small molecule inhibitors at 200 nM or with CD4 MAb at 5 μg/ml before incubation with the HIV-1 isolate GUN-lv. Infections were performed in duplicate, and error bars represent the standard errors of the means. Results are representative of those from at least two independent experiments.

...the efficient infection observed by the HIV and SIV strains shown in Fig. 5. The corresponding antibody isotype control, IgG1, had no effect on BMVEC infection. Infection of BMVECs therefore identifies the same subset of HIV-1, HIV-2, and SIV strains that infect GFAP⁺ astrocytes. Complete inhibition of BMVEC infection by vMIP-I, as well as the lack of inhibition by potent ligands for CCR5 and CXCR4, confirms that an alternative coreceptor is responsible for virus entry.

A subset of HIV and SIV isolates can infect Δ32/Δ32 CCR5 PBMCs with CXCR4 blocked by AMD3100. The experiments described above provide evidence that BMVECs and astrocytes naturally express alternative coreceptors that are functional for a subset of HIV-1 strains. Since these cells are not the major targets for HIV-1 in vivo, we tested whether the HIV and SIV isolates had the capacity to exploit an alternative coreceptor(s) on PBMCs. For this we used PBMCs prepared from an individual homozygous for the 32-bp deletion in CCR5 (Δ32/Δ32 CCR5) and blocked CXCR4 by treatment with AMD3100. The R5 HIV-1 isolate SF162 was unable to infect these cells, since they do not express CCR5 (data not shown). Infection of wt/wt CCR5 PBMCs by SF162 was not inhibited by the ligand AMD3100 (CXCR4) or vMIP-I (CCR5, GPR1, and CXCR6), either alone or in combination (Fig. 6A). Together these data demonstrate that SF162 infection of PBMCs is entirely CCR5 dependent. On Δ32/Δ32 CCR5 PBMCs, the X4 strain 2044 was efficiently inhibited by AMD3100 but not vMIP-I, although a slight delay in the rate of replication of this virus was noted (Fig. 6B). The HIV-1 isolate HAN-2 and...
HIV-2 TER replicated in the presence of AMD3100 (Fig. 6C and E). A second HIV-1 isolate, P1019, consistently replicated in the presence of AMD3100, albeit at low levels (Fig. 6D). Replication by each of these viruses was completely inhibited when cells were pretreated with vMIP-I in addition to AMD3100, yet pretreatment with 1309 in addition to AMD3100 had no effect on any of the isolates tested (data not shown). We also tested GUN-1v; however, this T-cell-line-adapted virus replicated poorly in both wt/wt and Δ32/Δ32 CCR5 PBMCs. GUN-1v replication eventually ensued after a long lag phase; however, the impact of adaptive changes occurring during this lag time on GUN-1v coreceptor use is unclear. GUN-1v data were therefore omitted from this study. The results described above thus show that the primary HIV-1 and HIV-2 strains that infect astrocytes and BMVECs are also able to exploit an alternative coreceptor on PBMCs, a major target for HIV infection in vivo.

Analysis of HIV and SIV receptor expression on untransformed cell types. RT-PCR was carried out on mRNA extracted from the three astrocyte cultures, BMVECs, Δ32/Δ32 PBMCs, and macrophages, to determine the pattern of HIV coreceptor expression. All mRNA preparations were positive for the housekeeping GAPDH gene (Fig. 7A). CD4 was found to be strongly expressed in Δ32/Δ32 CCR5 PBMCs and macrophages, as expected, but, surprisingly, also in the adult astrocyte culture 004.2 (Fig. 7B). BMVECs and the adult astrocyte culture 001A were very weakly positive for CD4, explaining the inhibition observed with anti-CD4 antibodies (Fig. 4B and 5B, respectively). A truncated CCR5 signal was detected for Δ32/Δ32 PBMCs and macrophages but not for any of the other cell cultures tested. CXCR6 expression was detected in PBMCs only. CXCR4 mRNA was expressed in Δ32/Δ32 PBMCs and macrophages, as well as in the fetal astrocyte culture F01. Although CCR3 and CCR8 were undetectable on Δ32/Δ32 PBMCs after 25 cycles, a signal was detected after amplification for 35 cycles (Fig. 7C). All other coreceptors screened for, including CCR3, CCR8, GPR1, and GPR15, were negative on all other primary cell cultures after 25 and 35 cycles (Fig. 7C). The orphan receptor RDC1 was detected in Δ32/Δ32 PBMCs, in the fetal F01 astrocytes, and weakly in the adult 004.2 astrocytes. In contrast to other studies (64), the parental NP2/CD4 cell line was also positive for this receptor. mRNA detection for each of the coreceptors tested here therefore did not correlate with HIV or SIV infection via the alternative coreceptor. We thus conclude that an as-yet-identified receptor for vMIP-I is partially (astrocytes) or wholly (BMVECs and PBMCs) responsible for infection of several untransformed cell cultures.
FIG. 7. mRNA expression of HIV and SIV receptors and coreceptors in primary cell types. RT-PCR was used to test for chemokine receptor mRNA expression. (A) Amplification of GAPDH served as a control for intact mRNA. (B) The presence of chemokine receptor mRNA in Δ32/Δ32 CCR5 PBMCs and macrophages, astrocytes, and BMVECs was determined, with mRNA from coreceptor-expressing cell lines NP2/CD4 (CCR5, CXCR4, CCR3, CCR8, and GPR1) and GHOST (CXCR6 and GPR15) acting as positive controls. NP2/CD4 cells naturally expressed RDC1. The primers used are shown in Table 2. Lanes MWM, 100-bp molecular size DNA ladder. Each experiment was carried out with negative (water) (lanes –) and positive (mRNA from cells expressing the specific receptor) (lanes +) controls, as well as with the parental NP2/CD4 cells as a cellular negative control. The absence of contaminating genomic DNA was confirmed by carrying out all RT-PCRs on untranscribed mRNA (data not shown).

DISCUSSION

A wide range of different seven-transmembrane G-protein-coupled chemokine receptors function as coreceptors for HIV infection of indicator cell lines in vitro. Many HIV-2 and SIV strains are particularly promiscuous for different coreceptors in such assays; however, HIV-1 strains can also use a variety of alternative coreceptors (16, 53), e.g., CCR3, CCR8, and
CXCR6. Although some of these coreceptors are expressed on CD4+ primary cells (1, 34, 61), there is little current evidence to suggest they are used by HIV-1 in vivo. Thus, the ability of particular HIV-1 strains to exploit alternative coreceptors for infection of cell lines in vitro does not provide a true indication of virus coreceptor use in vivo. We therefore analyzed several untransformed cell cultures for natural expression of functional alternative coreceptors that support HIV and SIV infection.

Astrocytes established from adult temporal lobectomy samples and untransformed BMVEC cultures do not express either CCR5 or CXCR4 and thus represent suitable target cells to test for the presence of alternative coreceptors. BMVECs and adult astrocytes expressing CD4 (via Ad-CD4) were resistant to infection by HIV and SIV strains that predominantly utilize CCR5 or CXCR4 alone. Both BMVECs and adult astrocytes were susceptible to infection by a subset of R5X4 HIV-1 strains, including two primary isolates (HAN-2 and P1019) as well as GUN-1v. GUN-1v is a variant virus isolated in vitro by its capacity to infect a wider range of host cells, including BT cells (human CD4+ meningioma-derived cells) (65, 71). GUN-1v infection of BMVECs was sensitive to inhibition by vMIP-I (an HHV8-encoded chemokine), suggesting that a receptor for this ligand is important for infection of these cells. In contrast, astrocyte infection by GUN-1v was reduced only approximately 50% by vMIP-I. This lack of complete inhibition by vMIP-I suggests either that the vMIP-I receptor (implicated for infection of BMVECs) is not solely responsible for astrocyte infection or that vMIP-I only weakly inhibits this receptor as expressed on astrocytes.

Infection of astrocytes was also reduced approximately 50% by the chemokines RANTES and eotaxin. These ligands each bind several chemokine receptors (CCR1, CCR3, CCR5, and CCR9 for RANTES and CCR3 and D6 for eotaxin), indicating a role for this subclass of receptor in the infection of astrocytes. Although CCR5 expression was not detected on these astrocytes, several groups have reported expression on astrocytes in situ in the brain, which is lost rapidly in culture (8, 29, 44). The lack of inhibition by the CCR5 small-molecule inhibitor TAK-779, as well as by the CCR5-specific MAb 2D7, confirms that the weak inhibition by RANTES was not due to the presence of low levels of CCR5.

There is evidence that astrocytes become persistently infected by HIV-1 in vivo, particularly in pediatric AIDS (57, 70, 74). Since R5 viruses are predominant in the brain (1, 37, 62), it is possible that these astrocytes are infected in vivo via CCR5. Nevertheless, the alternative coreceptor demonstrated on these CCR5-activated astrocytes may potentially confer infection of astrocyte subsets critical for brain homeostasis and thus have an impact on neuropathology. In this study we used MAbs that detect p24 as a marker for HIV-1 infection. Since p24 is a late gene product, our results show that the astrocyte infection observed here in vitro is not restricted to the early phase of viral replication and expression of early gene products, thus confirming the observations of Canki et al. (10).

Since BMVECs and astrocytes are not major targets for HIV-1 in vivo, we sought evidence for use of the alternative coreceptor on primary PBMCs. Other studies have demonstrated the ability of some SIV strains to infect PBMCs lacking CCR5 via an alternative coreceptor, in a donor-dependent manner (11, 78). In some instances CXCR6 was implicated as the coreceptor involved (79, 80). In confirmation, several HIV-1 and HIV-2 isolates identified here that exploit an alternative coreceptor(s) for BMVEC and astrocyte infection were able to replicate in PBMCs that lack CCR5, with CXCR4 blocked by AMD3100. The X4 isolate 204X4 was consistently sensitive to inhibition by AMD3100, although low-level residual replication was sometimes detected even in the presence of AMD3100. However, AMD3100-resistant replication in PBMCs by the isolates HAN-2, P1019, TER, and Man4 was not due to residual use of CXCR4, since such replication was inhibited by vMIP-I, a chemokine that does not interact with CXCR4 (20, 67). Our observations therefore provide evidence that an alternative coreceptor is active for infection of PBMCs by the HIV and SIV strains described.

The identity of the alternative coreceptor remains to be conclusively elucidated. A hallmark of the BMVEC and PBMC infection via an alternative coreceptor demonstrated here was the sensitivity to inhibition by vMIP-I. To date, vMIP-I has been reported to inhibit HIV infection mediated through CCR8 (20, 33) as well as GPR1 and CXCR6 (66). GUN-1v and the other HIV and SIV strains, identified here by their capacity to use alternative coreceptors, were able to use CCR8 and GPR1 (as well as other coreceptors) in addition to CCR5 and CXCR4 as coreceptors on cell lines. Furthermore, GUN-1v has previously been reported to use alternative coreceptors for infection of astroglial U87 cells, primary mesengial kidney cells, and BT cells. CCR8 was implicated as a coreceptor for BT infection (40) and GPR1 was implicated as a coreceptor for mesengial cell infection (73); however, inhibition by ligands to these coreceptors was not demonstrated. Our observations that the CCR8 ligand 1309 had no effect on the replication of any HIV or SIV isolate in BMVECs or Δ32/Δ32 PBMCs, alone or in combination with AMD3100, despite being able to inhibit infection of a CCR8-expressing indicator cell line (data not shown) implies that vMIP-I is able to inhibit HIV infection of PBMCs via another chemokine receptor.

We also attempted to correlate coreceptor mRNA detection in PBMCs, BMVECs, and astrocytes with infection by this subset of HIV and SIV strains. None of the presently known vMIP-I receptors (CCR8, GPR1, and CXCR6) were detected by RT-PCR in BMVECs. mRNA for CCR8 was detected in the Δ32/Δ32 PBMCs; however, the CCR8-specific chemokine 1309 had no effect on viral replication in either PBMCs or BMVECs. Δ32/Δ32 CCR5 PBMCs were also positive for expression of CXCR6, a coreceptor previously implicated as being responsible for replication of a maternal HIV-1 isolate (61, 79, 80). However, the CXCR6 ligand CXCL16 failed to block replication of vMIP-I-sensitive virus strains on Δ32/Δ32 CCR5 PBMCs even though infection of CXCR6-expressing cell lines was efficiently inhibited (data not shown). Finally, mRNA for GPR1 was not detected in either BMVECs or PBMCs. We therefore believe that an as-yet-unidentified vMIP-I receptor is responsible for the HIV and SIV infection of these cells. An alternative, albeit less likely, explanation is that vMIP-I induces down-modulation of a different receptor or induces intracellular signaling responses that negatively affect viral replication. However, previous studies have demonstrated that vMIP-I has no effect on chemokine-induced intracellular calcium signaling via the chemokine receptors CCR2, CCR4, CCR5, CCR7,
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CXCR2, CXCR3, and CXCR4 on PBMCs (20). Although
vMIP-I did desensitize CCR8-mediated signaling, its ineffec­
tiveness against such a large range of chemokine receptors
suggests that receptor desensitization is not the mechanism of
inhibition observed here.
All viruses that we found to use an alternative coreceptor(s)
are R5X4, with the exception of HIV-2 TER, an isolate that
readily acquires CXCR4 use in culture. We believe that an
alternative coreceptor(s) may be exploited late in disease in
addition to, or instead of, CXCR4 (like TER). The role of the
expansion in coreceptor use may therefore contribute to the
broadening of cell tropism late in disease, while a role in
neuropathogenesis should also be considered. Moreover, the
alternative coreceptor should be considered as a potential es­
cape route from new therapeutic CCR5 and CXCR4 inhibitors
currently undergoing clinical trials (34).
ACKNOWLEDGMENTS
This work was supported by grants from amFAR (02802-30-RG)
and the NIH (MH64408-01). S.J.W. is supported by Pfizer Global
Research and Development, United Kingdom. A M. is a recipient of a
Wellcome Trust Fellowship, United Kingdom. P.R.C. is an Elizabeth
Glaser Research Scientist.
We are grateful for the support, encouragement, and excellent dis­
cussion from Patrick Dorr and Manos Perros at Pfizer Global Re­
search and Development, Ltd. Hiroo Hoshino very generously pro­
vided the HIV-l strain G UN-lv and coreceptor-expressing NP2 cells.
We thank Brad Poulos at the Albert Einstein Human Tissue Reposi­
tory for fetal brain samples for astrocyte culture. We are grateful to the
EU Programme EVA/MRC Centralised Facility for AIDS Reagents,
NIBSC, United Kingdom (grant no. QLK2-CT-1999-00609 and
GP828102), and the AIDS Research and Reference Reagent Program,
Division of AIDS, NIAID, NIH, for reagents used in this study.
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