Enhancement of HIV-1 neutralisation by modulation of the virus envelope.

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Abstract

Despite over 25 years of research, the goal of producing an HIV-1 vaccine able to prevent infection has not been realised. Due to the limited immunogenicity of HIV-1 glycoproteins in vivo, this thesis explores the use of cholesterol depletion in increasing the antigenicity and immunogenicity of an whole-inactivated HIV-1 immunogen.

The primary aim was to demonstrate that cholesterol depletion of HIV-1 could enhance antibody binding and viral neutralisation. The laboratory-adapted strain, HIV-1\textsubscript{MN}, was utilised to demonstrate effective cholesterol depletion of virus using the compound methyl-β-cyclodextrin (MBCD). A range of MBCD concentrations were explored to assess the effect of removing cholesterol on viral infectivity, morphology and protein composition of the virus. A 1mM MBCD concentration, which effected a 50% reduction in viral envelope cholesterol with little impact on viral structure and protein composition whilst retaining viral infectivity, was chosen to explore the effect of cholesterol depletion on virus antibody binding and neutralisation. Removal of cholesterol from the viral envelope increased antibody binding and neutralisation using a number of monoclonal antibodies, soluble CD4 (sCD4) and HIV-1 positive patient antisera.

This concept was then extended to a pseudotyped primary isolate of HIV-1. Cholesterol depletion of this isolate demonstrated a more restricted enhancement of antibody binding and neutralisation of virus by monoclonal antibodies, sCD4 and homologous and heterologous HIV-1 infected patient antisera.

A number of inactivating agents were then explored in the aim of creating a cholesterol-depleted, whole inactivated HIV-1\textsubscript{MN} immunogen that retained key conformational gp160 epitopes. This immunogen was then tested in a mouse model to investigate whether cholesterol depletion could enhance the immunogenicity of whole-inactivated HIV-1\textsubscript{MN}. Although measurement of antibody responses from pooled mouse sera indicated significant enhancement of humoral responses to cholesterol-depleted, whole-inactivated HIV-1\textsubscript{MN}, analysis of individual mouse responses to immunisation yielded more variable results.
Acknowledgements

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I would also like to mention my family for always being there, ready with love and support.

Lastly, I would like to send my eternal thanks to Dr Dominic Rees-Roberts, who provided sound advice, patient proof-reading and for propping me up along the way.
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>°C</td>
<td>Degrees centrigrade</td>
</tr>
<tr>
<td>3’-OH</td>
<td>3-prime hydroxyl group</td>
</tr>
<tr>
<td>4-VP</td>
<td>4-vinylpyridine</td>
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<tr>
<td>ABCA-1</td>
<td>ATP-binding cassette transporter 1</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>AP</td>
<td>Alkaline phosphatase</td>
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<td>APOBEC3G</td>
<td>Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G</td>
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<td>Arg</td>
<td>Arginine</td>
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<tr>
<td>ARV</td>
<td>AIDS-associated retrovirus</td>
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<td>Asp</td>
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<td>AT-2</td>
<td>Aldrithriol-2</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AZT</td>
<td>Zidovudine – anti-retroviral drug</td>
</tr>
<tr>
<td>BAF</td>
<td>Barrier to auto-integration factor</td>
</tr>
<tr>
<td>BB</td>
<td>Blocking buffer</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacille Calmette-Guérin</td>
</tr>
<tr>
<td>BMV</td>
<td>Brome mosaic virus</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BPL</td>
<td>Beta-propiolactone</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>C</td>
<td>Conserved</td>
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<td>Carboxy-terminus</td>
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<tr>
<td>CA</td>
<td>Capsid</td>
</tr>
<tr>
<td>CAT</td>
<td>Chloramphenical acetyl transferase</td>
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<tr>
<td>CBP</td>
<td>CREB-binding protein</td>
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<tr>
<td>CCR</td>
<td>Chemokine (C-C) motif receptor</td>
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<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>CD4BS</td>
<td>CD4 binding site</td>
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<tr>
<td>CD4i</td>
<td>CD4 induced</td>
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</table>
CDK  Cyclin-dependent kinase

cDNA  Copy deoxy-ribonucleic acid

CDR  Complementarity-determining region

CHOL  Cholesterol

cm²  Centimetre squared

CMV  Cytomegalovirus

CO₂  Carbon dioxide

cpz  Chimpanzee

CREB  cAMP response element binding

CTL  Cytotoxic lymphocyte

CXCR  Chemokine (C-X-C) motif receptor

DC-SIGN  Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin

DIBA  Dithiobis benzamide

DIPE  Diisopropyl ether

DIS  Dimerisation initiation sequence

DL-PLG  Poly(DL-lactide-co-glycolide)

DMEM  Dulbecco’s minimal essential medium

DMMB  Dimethyl methylene blue

DMSO  Dimethyl sulfoxide

DNA  Deoxyribonucleic acid

dNTP  Deoxy-nucleotide

E.coli  Eschericia coli

EBV  Epstein-Barr virus

ECL  Enhanced chemiluminescence

EIAV  Equine infectious anaemia virus

ELISA  Enzyme-linked immunosorbent assay

env  Envelope gene

Env  Envelope protein

ESCRT  Endosomal sorting complex required for transport

FACS  Fluorescence –activated cell sorter

FCS  Fetal calf serum

FFU  Focus forming units
<table>
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<tr>
<th>Abbreviation</th>
<th>Acronym</th>
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<td></td>
<td>Feline immunodeficiency virus</td>
</tr>
<tr>
<td>gag</td>
<td></td>
<td>Group-specific antigen gene</td>
</tr>
<tr>
<td>Gag</td>
<td></td>
<td>Group-specific antigen protein</td>
</tr>
<tr>
<td>GalCer</td>
<td></td>
<td>Galactosylceramide</td>
</tr>
<tr>
<td>GCP</td>
<td></td>
<td>Good clinical practice</td>
</tr>
<tr>
<td>GM1</td>
<td></td>
<td>Monosialotetrahexosylganglioside</td>
</tr>
<tr>
<td>gp</td>
<td></td>
<td>Glycoprotein</td>
</tr>
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<td>GPCR</td>
<td></td>
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<td>GPLs</td>
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<td>Glycophospholipids</td>
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<tr>
<td>GSLs</td>
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<td>Glycosphingolipids</td>
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<td>HAART</td>
<td></td>
<td>Highly active anti-retroviral therapy</td>
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<td>HBMVECs</td>
<td></td>
<td>Human brain microvascular endothelial cells</td>
</tr>
<tr>
<td>HCl</td>
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<tr>
<td>HFV</td>
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<td>HIV</td>
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<td>HLA</td>
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<td>Human leukocyte antigen</td>
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<td>HMG1</td>
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<td>HPSG</td>
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<td>Human papilloma virus</td>
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<td>HR</td>
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<td>HRP</td>
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<td>IFNγ</td>
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<tr>
<td>IgA</td>
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<td>Immunoglobulin A</td>
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<td>IgG</td>
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<td>Immunoglobulin G</td>
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<tr>
<td>IgG-AP</td>
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<td>Immunoglobulin G conjugated to alkaline phosphatase</td>
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<td>IL-2</td>
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<td>Interleukin-2</td>
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<tr>
<td>IN</td>
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<td>Integrase</td>
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INA 1,5-iodonaphthylazide
IU International units
K₄Fe(CN)₆ Potassium ferrocyanide
KCl Potassium chloride
kDa Kilodalton
LAV Lymphadenopathy-associated virus
LEDGF Lens epithelial derived growth factor
LTNPs Long term non-progressor
LTR Long terminal repeat
LYS Lysine
MA Matrix
MAb Monoclonal antibody
MBCD Methyl-β-cyclodextrin
Mg²⁺ Magnesium ion
MgCl₂ Magnesium chloride
MHC Major histocompatibility complex
MHRP US military HIV-1 research program
MIP-1α Macrophage Inflammatory Protein 1 alpha
ml Millilitre
MLV Murine leukaemia virus
 mM Millimolar
MMuLV Moloney murine leukaemia virus
MPER Membrane proximal exposed region
mRNA Messenger ribonucleic acid
mU Milliunit
mV Millivolt
n Number
N'-terminus Amino-terminus
NaCl Sodium chloride
NaHCO₃ Sodium hydrogen carbonate
Nak kinase Nef-associated kinase
NaN₃ Sodium nitrate
NC Nucleocapsid
<table>
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<th>Definition</th>
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<td>Nef</td>
<td>Negative regulatory factor</td>
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<tr>
<td>NEM</td>
<td>$N$-ethylmaleimide</td>
</tr>
<tr>
<td>ng</td>
<td>Nanogram</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localisation signal</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometre</td>
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<tr>
<td>nM</td>
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<td>NOBA</td>
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<tr>
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<td>Overnight</td>
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<tr>
<td>$p$</td>
<td>$p$ value</td>
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<tr>
<td>p</td>
<td>Protein</td>
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<tr>
<td>Pak kinase</td>
<td>p21 activated kinase</td>
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<tr>
<td>PBMCs</td>
<td>Peripheral blood mononuclear cells</td>
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<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<td>PBS</td>
<td>Primer binding site</td>
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<tr>
<td>PBST</td>
<td>Phosphate-buffered saline with Tween-20</td>
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<tr>
<td>PC</td>
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</tr>
<tr>
<td>PCP</td>
<td>Pneumocystic pneumonia</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PHA</td>
<td>Phyto-haemagglutinin</td>
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<td>PHI</td>
<td>Primary HIV-1 infection</td>
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<tr>
<td>PI</td>
<td>Phosphatidylinositol</td>
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<td>PIC</td>
<td>Pre-integration complex</td>
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<td>PIP</td>
<td>Phosphatidylinositol-4-phosphate</td>
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<td>Pol</td>
<td>Polymerase gene</td>
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<td>Pol</td>
<td>Polymerase protein</td>
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<tr>
<td>polyA</td>
<td>Polyadenylation</td>
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<td>POPC</td>
<td>Palmitoyl-2-oleoylphosphatidylcholine</td>
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<td>PPMP (anti-retroviral)</td>
<td>(R)-9-[2-(phosphonomethoxy)propyl]adenine</td>
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<td>PPMP (GSL inhibitor)</td>
<td>1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol</td>
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<td>PPT</td>
<td>Polypurine tract</td>
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<td>PR</td>
<td>Protease</td>
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<td>P-TEFb</td>
<td>Positive transcription elongation factor b</td>
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<td>qRT-PCR</td>
<td>Quantitative reverse transcriptase polymerase chain reaction</td>
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<tr>
<td>R5</td>
<td>CCR5 using HIV-1 isolate</td>
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<tr>
<td>RANTES</td>
<td>regulated upon activation, normal T cell expressed and secreted</td>
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<td>Rev</td>
<td>Regulator of viral protein expression</td>
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<td>RNA</td>
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<td>Ribonuclease H</td>
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<td>RPMI</td>
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<td>RRE</td>
<td>Rev response element</td>
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<td>RT</td>
<td>Reverse transcriptase</td>
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<td>SAIDS</td>
<td>Simian acquired immunodeficiency syndrome</td>
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<td>SARS</td>
<td>Severe acute respiratory syndrome</td>
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<td>sCD4</td>
<td>Soluble CD4</td>
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<td>SDS</td>
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<td>SH3-like</td>
<td>Src-homology 3-like</td>
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<td>Simian immunodeficiency virus</td>
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<tr>
<td>SM</td>
<td>Sphingomyelin</td>
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<tr>
<td>SRV</td>
<td>Simian retrovirus</td>
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<tr>
<td>SU</td>
<td>Surface unit</td>
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<td>T cell</td>
<td>T lymphocyte</td>
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<td>TAR</td>
<td>Transactivation response region</td>
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<tr>
<td>Tat</td>
<td>Transactivator of transcription</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
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<tr>
<td>TCID_{50}</td>
<td>Tissue culture infectious dose 50%</td>
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<td>TFIID</td>
<td>Transcription factor IID</td>
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<td>T_{H} cell</td>
<td>T helper cell</td>
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<td>TiO_{2}</td>
<td>Titanium oxide</td>
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<tr>
<td>TM</td>
<td>Transmembrane</td>
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<td>TMB</td>
<td>Tetramethyl benzidine</td>
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<td>Taurine chloramine</td>
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<td>TRIM</td>
<td>Tripartite motif</td>
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<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
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<td>Description</td>
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<td>-------------</td>
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<tr>
<td>tRNA</td>
<td>Transfer ribonucleic acid</td>
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<td>Unit</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
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<td>Volume</td>
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<td>V</td>
<td>Volt</td>
</tr>
<tr>
<td>V</td>
<td>Variable</td>
</tr>
<tr>
<td>Vif</td>
<td>Viral infectivity factor</td>
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<tr>
<td>VLPs</td>
<td>Virion-like particles</td>
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<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>
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<td>VSV-G</td>
<td>Vesicular stomatitis virus G</td>
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<td>w</td>
<td>Weight</td>
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<td>WB</td>
<td>Wash buffer</td>
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<tr>
<td>x g</td>
<td>Times gravity</td>
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<td>CXCR4-using HIV-1 isolate</td>
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<td>X-gal</td>
<td>bromo-chloro-indolyl-galactopyranoside</td>
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<td>Zn$^{2+}$</td>
<td>Zinc ion</td>
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<td>β-gal</td>
<td>Beta-galactosidase</td>
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<td>Δ</td>
<td>Delta – deleted</td>
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<td>μg</td>
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</tr>
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<td>Microlitre</td>
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<td>µm</td>
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<td>Microunit</td>
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Chapter 1:

General Introduction
1.1 HIV-1 infection

With over 33 million people currently infected and 2.7 million new infections each year (UNAIDS, 2008), the Human Immunodeficiency Virus Type 1 (HIV-1) and Acquired Immunodeficiency Syndrome (AIDS) resulting from HIV-1 infection is a global problem (Figure 1.1). As HIV/AIDS-related deaths topped 3 million people in 2007 (UNAIDS, 2008), the need for a vaccine is acute, both in terms of health benefit and in economic terms.

Infection with HIV-1 occurs across the mucosa by exposure to semen, pre-seminal fluid and vaginal fluid. However, blood-to-blood transmission as a result of blood transfusion or the use of contaminated hypodermic needles is well documented. Vertical transmission from mother to child during pregnancy, childbirth or breast feeding is likely, but with the advent of HAART therapy vertical transmission has been reduced to less than 1% of infections in the U.K (Duong et al., 1999).

As HIV-1 cannot productively infect epithelial cells, the mechanism of HIV-1 transmission across the mucosa, in the absence of epithelial trauma, remains to be clarified. Epithelial attachment is thought to occur in a number of ways, including by direct interaction with sugar groups, such as heparin sulphate proteoglycan (HPSG), present on epithelial cell membranes (Ugolini et al., 1999). One possible mechanism of crossing the mucosal barrier, once trapped by the gp160 interaction with HPSG, is transcytosis. This is a process by which intact virions are transported through epithelial cells in vesicles (Yahi et al., 1992; Bomsel, 1997; Geijtenbeek et al., 2000), where the virus can infect immature dendritic cells or monocytes on the basal epithelium (Bomsel, 1997).

Cell-free virus within infectious secretions (semen and vaginal) is not transcytosed in the same way as virus derived from infected cells. In this instance, both mucosal trauma and transport across the epithelium by dendritic cells is important. Tschachler et al (1987) demonstrated that mature mucosal dendritic cells initiate primary infection by migrating to secondary lymphoid tissue to productively infect T cells (Tschachler et al., 1987). This occurs by high affinity binding of gp120 to dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) on the dendritic
Figure 1.1. Prevalence of HIV-1 worldwide.

33 million people living with HIV-1 in 2007
2.7 million new infections in 2007
2 million deaths due to AIDS in 2007

Figure 1.1 shows the prevalence of HIV-1 infection worldwide as a percentage of the population infected divided into geographical areas. Estimated total number of people living with HIV-1 worldwide, the number of new infections in 2007 and the total deaths due to AIDS in 2007 are shown in red (UNAIDS, 2008).
cell surface, followed by transportation of the attached virus to the lymph nodes where HIV-1 productively infects T cells (Geijtenbeek et al., 2000). Using this gp120 interaction, HIV-1 can remain associated with DC-SIGN for long periods of time (>4 days) without losing infectivity, while trafficking to the lymphoid tissue (~ 2 days) (Geijtenbeek et al., 2000).

Once across the mucosa, HIV-1 selectively infects key cells of the immune system, namely CD4+ T lymphocytes (CD4+ T cells), cells of the monocyte lineage and some dendritic cell populations. Primary or acute HIV-1 infection in vivo is accompanied by a reduction in CD4+ T cell numbers in the peripheral blood and a high plasma viral load (Figure 1.2), followed by seroconversion (Piatak et al., 1993). During this acute phase, 80-90% of those infected will experience ‘flu-like symptoms’ 2 to 4 weeks post-exposure accompanied by fever, lymphadenopathy, rash, myalgia, and malaise.

The acute period of infection is followed by clinical latency, where patients appear to be asymptomatic, with stable CD4+ T cell levels for between 2 weeks and 20 years (Figure 1.2). The rate of CD4+ T cell decline during this phase correlates with plasma virus load and may be important in the predicting clinical onset of AIDS (Gottlieb et al., 2002). In comparison with the rate of CD4+ T cell decline, viral load post-seroconversion is a more important prognostic marker for time to disease progression to AIDS (Mellors et al., 1997). Using defined viral load categories, percentage chance of progression to AIDS can be predicted. For example, 80% of patients with viral loads above 30,000 copies per millilitre (ml) with progress to AIDS within 6 years (Mellors et al., 1997).

Acquired immunodeficiency syndrome is diagnosed at a point when patient CD4+ T cells drop below 200 cells per microlitre of blood, which is associated with loss of cell-mediated immune function. In a study conducted in Uganda, patients with HIV-1 but not undergoing HAART therapy had a median survival time from the onset of AIDS to death of 10 years (Morgan et al., 2002). After the onset of AIDS, HIV-1 patients suffer from weight loss and a variety of opportunistic infections including Candidiasis, tuberculosis, Pneumocystis pneumonia (PCP), Epstein-Barr virus (EBV), cytomegalovirus (CMV), respiratory tract infections and cancers such as Kaposi’s sarcoma or B cell lymphomas.
Figure 1.2. Cellular immune responses during HIV-1 infection.

Figure 1.2. shows the CD4$^+$ T cell count throughout the time course of HIV-1 infection along with the respective viral load levels in a generalised diagram. During acute infection, CD4$^+$ T cell counts are reduced by around 50% and this corresponds to the peak of viraemia. During clinical latency (chronic infection stage) the viral load establishes a set-point and allows the recovery of CD4$^+$ T cells, but not to the levels observed before infection. During clinical latency the CD4$^+$ T cell count slowly declines and when these cells fall below 200 cells/mm$^3$ clinical symptoms of AIDS may appear and viral load becomes uncontrolled, eventually leading to death. Diagram modified from (Pantaleo et al., 1993).
1.2 The discovery of HIV

In the early 1980’s, increasing numbers of patients presenting with opportunistic infections, such as *Pneumocystis* pneumonia and the rare cancer Kaposi’s sarcoma were observed. This unknown disease was characterised by immune dysfunction resulting from the depletion of CD4$^+$ T cells in the peripheral blood. Although this infection was first associated with homosexual populations, prevalence of this disease was later found in groups of haemophiliacs, intravenous drug users and heterosexual Haitians (Selik et al., 1984). With high mortality rates, identification of the cause of this infection was critical.

A virus was first isolated from an asymptomatic patient in 1983 and was suggested to be the infectious agent involved in the cause of AIDS (Barre-Sinoussi et al., 1983). This virus, exhibited reverse transcriptase activity and was identified as a retrovirus distinct from HTLV-1 and was named lymphadenopathy-associated virus (LAV) (Barre-Sinoussi et al., 1983).

In the following year, a similar human retrovirus was isolated from an AIDS patient by Gallo et al. (1984) and designated human T cell leukaemia virus type III (HTLV-III). Gallo’s group obtained convincing evidence of a serological link between patients exposed to LAV and HTLV-III viruses and immunodeficient patients (Gallo et al., 1984; Popovic et al., 1984; Sarngadharan et al., 1984; Schupbach et al., 1984). Only later were LAV and HTLV-III shown to be the same isolate (Chang et al., 1993). Levy et al. (1984) had also isolated a similar retrovirus from both AIDS patients and asymptomatic individuals at risk of infection, which was named AIDS-associated retrovirus or ARV (Levy et al., 1984). This work confirmed that symptomatic and asymptomatic forms of the disease existed, suggesting possible latency of the virus. In 1986, these isolates were renamed HIV after characterisation of the LAV/HTLV-III and ARV viruses into the *Lentivirus* genus of the *Retroviridae* family (Ratner et al., 1985; Wain-Hobson et al., 1985), based on viral reverse transcriptase activity and the presentation of a slow, latent disease targeting haematopoietic cells.

A genetically related, but immunologically distinctive retrovirus was isolated from AIDS patients in West Africa in 1986 and was subsequently named HIV-2 (Clavel et
The genome of the HIV-2 virus has 42% overall homology with HIV-1 (Guyader et al., 1987), but possesses extended long terminal repeat (LTR) regions and encodes an extra protein, viral protein X (Vpx), responsible for nuclear import of viral DNA (Tristem et al., 1992; Sharp et al., 1996). Unlike HIV-1, HIV-2 is more closely related to the simian immunodeficiency virus (SIV) from sooty mangabeyes, SIV<sub>SM</sub>, exhibiting 75% genomic homology (Hirsch et al., 1989; Gao et al., 1992). In comparison with the disease course for HIV-1, HIV-2 infections are often asymptomatic for longer with delayed progression to AIDS.

### 1.3 The origin and genetic diversity of HIV-1

HIV-1 originated from cross-species transmission events of the SIV from chimpanzees, SIV<sub>cpz</sub>, into humans over 50 years ago (Zhu et al., 1998; Gao et al., 1999). Lines of evidence of zoonotic transmission of SIV<sub>cpz</sub> include the similarities in genome organisation and phylogenetics compared with HIV-1. Moreover, occurrence of SIV<sub>cpz</sub> in the natural host and its geographic distribution coincide with the emergence of infection, as well as possible routes of transmission, namely through the bush meat trade. All of these factors point to the chimpanzee species P. t. troglodytes as the natural reservoir of SIV<sub>cpz</sub> and the source of HIV-1 infection (Gao et al., 1999).

A study of genetic divergence of HIV-1 in humans has led to the evolution of three main groups, main (M), outliers (O) and non-M, non-O (N) (Sharp et al., 1999). Group M represents the majority of all global isolates and is sub-divided by further quasispecies variation into 9 clades A, B, C, D, F, G, H, J and K (Figure 1.3), clades A and F are further divided into types 1 and 2 (Robertson et al., 2000). The most common strains seen throughout Europe and the U.S.A. are the group M, B clade viruses (Sharp et al., 1999; Thomson et al., 2002). The worldwide clade prevalence and epidemiology is detailed in Table 1.1.
Figure 1.3. The diversity and evolution of HIV and SIV.

Figure 1.3 shows the genetic diversity and evolution of the HIV and SIV subtypes. HIV-1 is closely related to SIV<sub>cpz</sub>, while HIV-2 is more closely related to SIV<sub>sm</sub>. Genetic divergence of HIV-1 in humans has led to the evolution of three main subtypes: M, N, and O (Sharp <i>et al.</i>, 1999). Group M represents the majority of all global isolates and is sub-divided into clades A, B, C, D, F, G, H, J and K, classifying further genetic variation (Clade K is not shown). Figure adapted from (Knipe <i>et al.</i>, 2001).
Table 1.1. Worldwide distribution of HIV-1 groups and clades by geographical region.

<table>
<thead>
<tr>
<th>Geographical area</th>
<th>Group prevalence</th>
<th>Clade prevalence</th>
<th># of people living with HIV</th>
<th># of new infections</th>
<th># of AIDS related deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sub-Saharan Africa</td>
<td>M, N, O</td>
<td>Group M: clades C, A, F, G</td>
<td>22,000,000</td>
<td>1,900,000</td>
<td>2,000,000</td>
</tr>
<tr>
<td>Asia</td>
<td>M</td>
<td>B, C, F</td>
<td>5,000,000</td>
<td>380,000</td>
<td>380,000</td>
</tr>
<tr>
<td>Eastern Europe and Central Asia</td>
<td>M</td>
<td>A, B</td>
<td>1,500,000</td>
<td>110,000</td>
<td>58,000</td>
</tr>
<tr>
<td>Middle East and North Africa</td>
<td>M</td>
<td>C, D</td>
<td>380,000</td>
<td>40,000</td>
<td>-</td>
</tr>
<tr>
<td>Oceania</td>
<td>M</td>
<td>B</td>
<td>74,000</td>
<td>13,000</td>
<td>-</td>
</tr>
<tr>
<td>Caribbean</td>
<td>M</td>
<td>B, C</td>
<td>230,000</td>
<td>20,000</td>
<td>14,000</td>
</tr>
<tr>
<td>Latin America</td>
<td>M</td>
<td>B, C</td>
<td>1,700,000</td>
<td>140,000</td>
<td>63,000</td>
</tr>
<tr>
<td>North America, Western and Central Europe</td>
<td>M</td>
<td>B</td>
<td>2,000,000</td>
<td>81,000</td>
<td>31,000</td>
</tr>
</tbody>
</table>

1 – 62% of new infections are transmitted through injecting drug use
2 – 1,200,000 people infected in the United States of America

Table 1.1. The distribution of HIV-1 subtypes worldwide by geographical area. Estimated total number of people living with HIV-1, the number of new infections and the total deaths due to AIDS in 2007 are detailed by geographical region (UNAIDS, 2007).
1.4 HIV-1 morphology

HIV-1 has been shown by electron microscopy to be an enveloped, spherical to pleomorphic virus, approximately 100nm in diameter (Figure 1.4) (Gelderblom et al., 1987; Stannard et al., 1987). Mature HIV-1 particles contain a dimerised diploid positive (+) single stranded (ss) RNA genome bound to nucleocapsid protein (NC, p7). The conical viral core is constructed of capsid protein (CA, p24) and contains the NC:genome complex along with the virally encoded reverse transcriptase (RT) and integrase (IN) enzymes. The viral protease (PR) enzyme is also present in the mature virion. The matrix protein (MA, p17) lines a host derived envelope into which the viral glycoprotein receptor, gp160, is embedded. As the viral envelope is host derived, proteins such as major histocompatibility complex (MHC) or human leukocyte antigens (HLA) antigens are also incorporated into the envelope. The structure of the genome as well as the structure and function of all HIV-1 proteins are discussed in sections 1.5 and 1.7, respectively.

1.5 The HIV-1 genome

The HIV-1 proviral genome is approximately 9,700 base pairs (bp) in length and is made up of 3 main genes common to all retroviruses: gag, pol and env (Knipe et al., 2001). These genes encode polyproteins that are post-translationally cleaved to create the structural proteins, viral enzymes and surface receptor glycoproteins, respectively (details of the structure and function these proteins can be found in section 1.7.3). Unlike simple retroviruses, for example murine leukaemia virus (MLV), the HIV-1 genome encodes six accessory proteins (Vif, Vpr, Tat, Vpu, Rev and Nef) important in viral replication (details of the accessory proteins and their function can be found on in Section 1.7.4).

The viral genome (Figure 1.5) exists as a dimer of two positive single strands of RNA joined at their 5’ end by a dimer linkage sequence (Darlix et al., 1990; Paillart et al., 1996). The existence of two genome copies provides an alternative template in case of genomic damage (Balakrishnan et al., 2003). Dimerisation is co-ordinated by a dimerisation initiation sequence (DIS) at the 5’end of the viral genome consisting of a palindromic sequence (GCGCGC) allowing base pairing of individual RNA molecules.
**Figure 1.4.** HIV-1 morphology and virion structure

**A**

![Diagram](image)

- p7 (nucleocapsid, NC)
- gp41 (TM)
- Integrase (IN)
- Reverse transcriptase (RT)
- p24 (capsid, CA)
- Protease (PR)
- p17 (matrix, MA)
- gp120 (SU)

**B**

![Electron micrograph](image)

**Figure 1.4.** shows an HIV-1 virion (A) containing a dimerised diploid positive single stranded RNA genome encapsulated in three layers of structural proteins (NC, CA and MA). The virally encoded enzymes (RT, IN and PR) are found within the virus. The matrix protein (p17) lines a host derived envelope into which the viral glycoprotein receptor, gp160, is embedded. As the viral envelope is host derived, proteins such as MHC or HLA antigens are also incorporated into the envelope. The associated key shows all viral components depicted. (B) Electron micrograph of HIV-1 virions showing spherical to pleomorphic particles approximately 100nm in diameter, with a dense central core (p24 capsid). Image taken from [www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/).
Figure 1.5. The HIV-1 genome.

Figure 1.5 shows the organisation of the HIV-1 genome. The HIV-1 genome is approximately 9,700 bp in length and encodes 9 genes between long-terminal repeat (LTR) sequences at each end of the genome. Genes are translated into 3 groups of proteins, the Gag structural proteins (blue), the surface glycoproteins (red), the enzymes (purple) and the accessory proteins (in various colours). The primer binding site (PBS) and polypurine tracts (PPT) are important in reverse transcription. This process is discussed in more detail in Section 1.6.3.
The genome is flanked by long terminal repeat (LTR) regions at each end of the RNA.

### 1.6 HIV-1 replication

#### 1.6.1 HIV-1 receptors

The tropism of HIV-1 for T cells and primary immune cells of the monocyte lineage led to the discovery of the primary HIV-1 receptor in 1984, this was identified as the differentiation antigen, CD4 (Figure 1.6). The CD4 molecule was confirmed as an HIV-1 receptor by the co-immunoprecipitation of gp120-CD4 complexes (Dalgleish et al., 1984; McDougal et al., 1985) and observations that denaturation of the HIV-1 envelope protein disrupted the gp120-CD4 interaction (McDougal et al., 1986). Furthermore, otherwise uninfectable human cells were rendered susceptible to HIV-1 infection on transfection with CD4 sequences confirming CD4 as the HIV-1 receptor (Maddon et al., 1986). Figure 1.6 shows the structure of the HIV-1 receptors.

Feng et al. (1996) identified the first HIV-1 co-receptor by infecting non-permissive cells with a vaccinia virus library containing cDNA from permissive cells. This receptor was putatively named ‘fusin’, but on identification of the protein function was renamed CXCR4 (chemokine CXC motif receptor 4). The CXCR4 receptor is a G-protein coupled receptor (GPCR) and is primarily used by laboratory-adapted HIV-1 strains during membrane fusion (Feng et al., 1996). Therefore, a corresponding co-receptor for primary, macrophage-tropic viruses was hypothesised.

The chemokines RANTES, MIP-1α and β were found to be secreted by CD8+ T cells and peripheral blood mononuclear cells (PBMCs, although to a lesser extent) during in vitro infection and could inhibit viral fusion (Cocchi et al., 1995; Alkhatib et al., 1996). Initially hypothesised as an antiviral immune response, the effect of these chemokines during HIV-1 infection in vivo was thought to be of little physiological relevance (Cocchi et al., 1995). Extention of this study showed that RANTES, MIP-1α and β specifically inhibited macrophage-tropic viruses only and led to the identification of CCR5 (also a seven transmembrane spanning GPCR) as an HIV-1 co-receptor (Figure 1.5) (Alkhatib et al., 1996). Transfection of non-permissive cells with CD4 and CCR5
Figure 1.6. The figure shows the structure of the HIV-1 gp160 glycoprotein, the primary HIV-1 receptor, CD4, and the co-receptor CCR5. The gp160 protein is made up of a trimer of gp120:gp41 dimers, with gp41 forming the transmembrane domain and gp120, projecting outwards from the surface of the virion. The surface glycoprotein, gp120, interacts with the HIV-1 receptors CD4 and CCR5. The GPCR CXCR4, which is not shown in this diagram, has a similar structure to CCR5. Image taken from: http://www3.niaid.nih.gov/labs/aboutlabs/VRC/StructuralVirologyLaboratory/ (Wyatt)
allowed fusion of macrophage-tropic HIV-1 primary isolates BAL (Gartner et al., 1986), SF (Levy et al., 1984) and JRFL (O’Brien et al., 1990), but not the laboratory-adapted CXCR4-using strains LAV (Montagnier et al., 1984), IIIB or RF (Popovic et al., 1984) confirming CCR5 as the HIV-1 co-receptor for primary, macrophage-tropic isolates (Alkhatib et al., 1996).

Viral gp120 interacts with both CCR5 and CD4 (Figure 1.4), but in the absence of CD4 the affinity of the co-receptor for gp120 is reduced (Wu et al., 1996). This proposed a mechanism by which initial CD4 binding to gp120 creates a high affinity binding site for the HIV-1 co-receptors by altering protein conformation (Dalgleish et al., 1984; Trkola et al., 1996; Wu et al., 1996; Hill et al., 1997).

Specific regions within gp120 have been implicated in the interaction with viral co-receptor on the cell, for example the V3 region, which is important in viral tropism and co-receptor interaction. This has been shown by switching the co-receptor usage of HIV-1 isolates by changing V3 domains between viral strains (Hoffman et al., 1999).

All HIV-1 isolates are characterised according to co-receptor usage. The CCR5 (R5)-using strains are macrophage tropic, whereas CXCR4 (X4)-using isolates are commonly T cell line adapted. However, exceptions to these classifications and co-receptor usage have been found. A cloned isolate derived from HIV-1_{IIIB} (named HIV-1_{IIIBX}) has been extensively phenotyped due to its ability to utilise both the CXCR4 and CCR5 co-receptors, resulting in infection kinetics that are cytopathic and syncytium-inducing in primary T cells and macrophages (Collman et al., 1992).

Primary receptor (CD4) independent strains of HIV-1, HIV-2 and SIV have also been characterised (Endres et al., 1996; Hoffman et al., 1999). These isolates possess glycoproteins that are able to bind directly to cells expressing co-receptors only. The binding of some neutralising antibodies is induced by sCD4 (for example, 17b or 48D which are discussed in section 1.9.3) and these antibodies bind more readily to CD4-independent isolates illustrating the triggered gp120 state, with the conserved co-receptor binding site exposed (Hoffman et al., 1999). This also highlights the masked nature of the co-receptor binding site in native gp120 oligomers (Hoffman et al., 1999).
These isolates constitute attractive vaccine candidates for eliciting cross-reactive neutralising antibodies, like 17b or 48D, but these antibodies may be ineffective against the native virus (Hoffman et al., 1999).

Co-receptor usage has been linked to disease progression to AIDS. Viruses isolated from early infection classically use the CCR5 co-receptor and mediate macrophage-tropic productive infection with low levels of cytotoxicity (Schuitemaker et al., 1991; Schuitemaker et al., 1992; Endres et al., 1996). Upon progression to AIDS, viral isolates expand their co-receptor usage to incorporate CXCR4, CCR2 and CCR3, leading to highly cytopathic, primary isolates adapted to T cell infection (Collman et al., 1992; Connor et al., 1997).

Discovery of the HIV-1 co-receptors has led to the investigation of chemokines as inhibitors of HIV-1 infection. In studies of highly-exposed uninfected individuals, increased CCR5-binding chemokine (RANTES, MIP1α and β) secretion from CD4+ T cells may result in the possible protection from infection or prolonged progression to AIDS in these populations (Saha et al., 1998). Reduced infection frequency in heterozygous ∆CCR5 individuals confers natural HIV-1 resistance amongst populations as a result of co-receptor deficiency (Dean et al., 1996). Individuals with homologous ∆CCR5 genes have shown to be protected against HIV-1 infection. These case studies illustrate the possible use of CCR5 inhibitors in the treatment of HIV-1 in both prophylactic and therapeutic settings (Liu et al., 1996).

1.6.2 Viral-cell membrane fusion

Direct fusion with the cell membrane represents the primary method of viral entry for HIV-1, however, endocytosis has been observed with some HIV-1 isolates. Studies from the late 1980’s demonstrated that neutralising the acidic environment of cellular endosomes did not affect HIV-1 fusion (Stein et al., 1987; McClure et al., 1988). This contradicts the acidic endosomal environment that is essential for the infectivity of pH-dependent fusogenic enveloped viruses (Stein et al., 1987; McClure et al., 1988). In contrast, work by Fackler et al. (2000), showed that the HIV-1 isolate, SF2, is endocytosed at a low pH (pH 5) (Fackler et al., 2000). Moreover, the use of endocytosis inhibitors reduces viral infectivity illustrating this alternative endocytosis
pathway for viral cell entry (Fackler et al., 2000). Therefore, virus entry into a cell by endocytosis has been documented to occur through both pH-independent and pH-dependent mechanisms (Stein et al., 1987; McClure et al., 1988; Fackler et al., 2000; Miyauchi et al., 2009). The advantage of a receptor-independent form of cell entry increases the range of HIV-1 susceptible cells to those without the CD4 antigen, for example, fibroblasts or glial cells (Fackler et al., 2000). Despite this evidence, endocytotic entry can lead to inactivation or degradation of viral particles in lysosomes and, therefore, the true relevance of endocytotic cell entry by HIV-1 remains undetermined (Gomez et al., 2005).

Direct fusion of virus occurs in an ordered series of events that is initiated by HIV-1 interaction with the primary receptor, CD4 (Dalgleish et al., 1984; McDougal et al., 1985; McDougal et al., 1986). This exposes a conserved co-receptor binding site for CXCR4 or CCR5, depending on isolate tropism (Schuitemaker et al., 1991; Schuitemaker et al., 1992; Wu et al., 1996; Liu et al., 2008). Interactions with four to six receptors are required for cell fusion and this is facilitated by the concentration of the receptors within the cholesterol-rich lipid raft regions of the cell membrane (Layne et al., 1990; Kuhmann et al., 2000; Steffens et al., 2004; Gomez et al., 2005). Lipid rafts are important during cell fusion, as removal of cholesterol from either the virus or the cell membrane ablates viral infectivity by inhibiting membrane fusion (Viard et al., 2002; Steffens et al., 2004). It has also been observed that cholesterol is important for maintaining CCR5 receptor conformation and function (Steffens et al., 2004; Gomez et al., 2005).

Association of the gp120-CD4 complex with a co-receptor promotes conformational changes in gp41, exposing a hydrophobic fusion peptide which subsequently inserts into the cell membrane (Chan et al., 1998; Steffens et al., 2004). Both co-receptor and fusion peptide interaction with the membrane allows the generation of a 6-helix bundle, which is derived from the triple-stranded coiled-coil N'-terminal protein formation of native trimeric gp41 (Dong et al., 2001). Each monomer of a gp41 trimer folds, via a hairpin bend, into this 6-helix bundle formation bringing the two membranes into close proximity and releasing sufficient energy to allow lipid mixing (Munoz-Barroso et al., 1998; Melikyan et al., 2000). Figure 1.7 illustrates the steps that take place during fusion. A lag time between HIV-1 attachment to the cell and membrane fusion has
Figure 1.7. Fusion of the HIV-1 envelope with the cell membrane. 1 and 2: Direct fusion of virus is initiated by interaction of gp120 with the primary receptor, CD4. This exposes a conserved co-receptor binding site for CXCR4 or CCR5. 3: Association of the gp120-CD4 complex with a co-receptor promotes conformational changes in gp41, exposing the hydrophobic fusion peptide which subsequently inserts into the cell membrane. 3 and 4: Both co-receptor and membrane interaction via the fusion peptide allows the generation of a 6-helix bundle, bringing the two membranes into close proximity and releasing sufficient energy to allow lipid mixing. Diagram is adapted from (Caffrey et al., 1998; Louis et al., 2001)
been observed and is due to this helical gp41 rearrangement acting as the rate-limiting step (Melikyan et al., 2000; Steffens et al., 2004).

An understanding of the events occurring during membrane fusion has facilitated the development of fusion inhibitors (Kilby et al., 1998; Munoz-Barroso et al., 1998). Small peptide fragments of gp41 have been shown to inhibit HIV-1 infectivity by blocking membrane fusion (Munoz-Barroso et al., 1998). Binding of these peptides to the exposed gp41 triple stranded coiled-coil inhibits the conformational changes in gp41 involved in forming the 6-helix bundle (Munoz-Barroso et al., 1998). These initial experiments with peptide fusion inhibitors led to the first HAART fusion inhibitor drug, T20, now widely used in HIV-1 therapy (Kilby et al., 1998).

1.6.3 Post-entry events

Uncoating

The first step in viral replication after membrane fusion is viral uncoating, the process of which is still largely unknown. Indicatively, it must involve the disruption of the CA core allowing for genomic RNA reverse transcription. Substitution of three key serine residues in CA (Ser 109, Ser 149 and Ser 178) reduced the uncoating of purified core particles and inhibited reverse transcription, indicating a role for the CA protein itself in this process (Wacharapornin et al., 2007). These serine residues are phosphorylation sites and, therefore, phosphorylation of CA by host factors may cause changes in protein conformation which disrupt the viral core (Amella et al., 2005; Wacharapornin et al., 2007).

Reverse transcription

Once the viral RNA is uncoated, the process of reverse transcription can occur in the cytoplasm. This is mediated by the heterodimeric RT protein (p66/p51), which is encoded by the viral genome and packaged into the progeny virions. The RT enzyme has both RNA-dependent DNA polymerase (p66) and RNase H activity (p51) activity (Ferris et al., 1990; Gutierrez-Rivas et al., 2001). Viral linear DNA can be detected 4
hours post-infection and involves both copies of the genome (Panganiban et al., 1988; Kim et al., 1989; Temin, 1993).

A tRNA\textsuperscript{LYS} primer is prebound to the primer binding site located in the 5’ LTR (Figure 1.8) and binds RT upon infection of a cell to initiate production of the DNA minus (-) strand (Temin, 1993). Minus strand synthesis then occurs through the viral 5’ LTR before transferring to the 3’ end of the RNA genome (Temin, 1993). At this point the synthesised DNA can translocate to the 3’ end of the same RNA molecule (intramolecular) or to the same site in the second genome copy (intermolecular) (Panganiban et al., 1988; Temin, 1993). It has been shown that both RNA genomes are used at this stage and therefore the (-) strand DNA binds intermolecularly to continue reverse transcription (Panganiban et al., 1988). The fragment of DNA synthesised from the 5’ LTR region acts as a primer to copy the remaining sequence of the genome to complete the (-) strand DNA (Temin, 1993). The RNase H activity of RT then cleaves the RNA template just after the PPT region (Figure 1.3) and degrades the RNA in the 5’ direction leaving the 3’ end of the PPT to serve as a primer for positive (+) strand DNA synthesis (Temin, 1993). Upon synthesis throughout the LTR and PBS site the DNA molecule anneals intramolecularly to the 3’ end of the (-) DNA strand where DNA synthesis can complete the cDNA sequence (Temin, 1993). The process is illustrated schematically in Figure 1.8.

**Nuclear translocation**

Immediately after reverse transcription, proviral DNA is incorporated into the pre-integration complex (PIC). Unlike oncoretroviruses, the HIV-1 virus is able to infect non-dividing cells due to its ability to import the integration machinery (coupled to viral genomic cDNA) into the nucleus (Marin et al., 2003; Zhang et al., 2003). Nuclear import occurs by interaction of nuclear localisation signals (NLS) possessed by many component proteins of the PIC with importin/karyopherin-α nuclear import proteins in the nuclear envelope (Gallay et al., 1997). The plus-strand overlap created during reverse transcription has also been associated with nuclear import, as mutants lacking this structure accumulate in the cytoplasm of infected cells as unintegrated linear DNA forms, however, this could equally be due to the inability to form the PIC (Zennou et al., 2000).
Reverse transcription occurs in a 3’ to 5’ direction and is initiated by the hybridisation of a tRNA$^{\text{Lys}}$ (rose) molecule to the PB (1). This provides a hydroxyl-group for reverse transcription to occur through the 5’ LTR (U5 and R) region (1). As reverse transcription takes place, the ssRNA is degraded by the RNase H activity of RT leaving a ssDNA 3’ end (1). The ssDNA-tRNA hybrid then transfers to the 3’-end of the genome template and full synthesis of the DNA strand (2). The ssRNA molecule is then degraded by RNase H from the 5’ end to the central PP, which serves as the primer for the synthesis of the second DNA strand (3). The presence of the tRNA molecule allows reverse transcription through the complementary PB (3). After degradation of the tRNA, the PB sites of the DNA molecules align and the complex circularises (4). The RT molecule then completes the DNA duplex by synthesising the rest of the second DNA molecule followed by the R and U3 region on the first strand to complete the DNA molecule complete with LTR regions at both ends of the provirus (5). Diagram adapted from (Mudrow et al., 2003).
The PIC is a high molecular weight protein complex, the composition of which is being largely undetermined (Chen et al., 1998). The viral integrase (IN) protein is a pivotal component of the PIC, mediating the integration of proviral DNA into the host genome (Farnet et al., 1991), as well as containing an atypical NLS (Gallay et al., 1997). However, functional IN is not required for nuclear import, suggesting the NLS alone is not required in this process (Bukrinsky et al., 1992). The ends of the cDNA are bound to IN at consensus sequences in the terminal portions of the provirus, protecting it from exonuclease, but not endonuclease attack (Bushman et al., 1991; Miller et al., 1997; Esposito et al., 1998). Other viral proteins have been associated with the PIC, for example the viral protein R (Vpr) possesses an NLS sequence, associated with PIC nuclear import (Vodicka et al., 1998).

Integration

Integration also requires the participation of cellular proteins, as adding cellular extracts to purified IN complexes aids efficient cDNA integration by purified IN in vitro (Chen et al., 1998). The cellular factors BAF (barrier-to-autointegration factor), HMG1(γ) (high mobility group 1γ) and LEDGF (lens epithelial derived growth factor) have all been identified as IN cofactors, facilitating proviral integration (Miller et al., 1997; Chen et al., 1998; McKee et al., 2008). These factors act as tethers to couple the IN complex with host DNA (McKee et al., 2008).

Integration is initiated by the removal of 2 nucleotides from the 3’ ends of the proviral DNA adjacent to a CA base pair conserved sequence, termed 3’ end processing (Bushman et al., 1990; Esposito et al., 1998). The exposed hydroxyl groups can then be used in direct trans-esterification (strand transfer) to the 5’ ends of a double stranded break in the host DNA (Bushman et al., 1990; Esposito et al., 1998). The remaining unpaired nucleotides at the 5’ end of the proviral DNA are removed and single-stranded DNA end segments of the viral DNA are joined to the host DNA by cellular DNA repair enzymes (Bushman et al., 1990; Esposito et al., 1998).
1.6.4 Viral assembly, budding and maturation

Viral proteins, produced by the cellular biochemical machinery assemble at the plasma membrane where protein multimerisation creates a curvature in the cell membrane that pinches off to form a progeny virion (Cimarelli et al., 2002). The Gag polyprotein is pivotal in viral assembly, as purified protein alone can form virion-like particles (VLPs) resulting from Gag multimerisation (Cimarelli et al., 2002; Ako-Adjei et al., 2005). Insertion of Gag into the plasma membrane is mediated by a myristol residue within the protein, which associates with the membrane to facilitate clustering of basic amino acids that interact with the inner lipid leaflet (Cimarelli et al., 2002). Gag multimerisation is co-ordinated by CA-CA, MA-MA and NC-NC interactions within the polyprotein and is associated with lipid raft structures (Cimarelli et al., 2002).

Envelope proteins are assembled into new virions by interactions between the gp41 monomer cytoplasmic tail (unusually long at 150 amino acids) and the MA portion of the p55 Gag polyprotein (Murakami et al., 2000; Murakami et al., 2000). Envelope gp41 also contains a cholesterol binding motif at amino acid residues 678-683 (LWYIK), promoting incorporation of Env into lipid rafts during virion assembly (Vincent et al., 2002).

The viral genome is efficiently packaged into the budding virus through interactions with the NC protein. Nucleocapsid binds to the genome encapsidation signal (ψ) within viral genomic transcripts allowing the association of the viral genome with assembling virions (Morellet et al., 1992; Mark-Danieli et al., 2005). A small amount of Vif has also been detected in progeny virions and this is thought to be as a result of association with viral RNA (Khan et al., 2001). The viral enzymes are incorporated into the budding virus most likely as a result of their production as a Gag-Pol precursor protein, mediated by Gag multimerisation (Cimarelli et al., 2002). The enzymes encoded by HIV-1 must be packaged into the progeny virions as they are essential for viral replication, as no cellular equivalents exist.
On accumulation of the relevant viral proteins at the infected cell surface, viral budding and maturation occur in tandem. The p6 protein present at the C’-terminal of the Gag polyprotein is essential for virus particle production as it enables release of the progeny virion (Gottlinger et al., 1991; Huang et al., 1995). Two peptide sequences within the p6, the PTAP or PxxP motif (Proline-Threonine-Alanine-Proline) and the YPLSTL sequence, are critical for virus release (Huang et al., 1995; Morita et al., 2004; Bieniasz, 2006). These two regions in p6 provide docking sites for the recruitment of ESCRT (endosomal sorting complex required for transport) and ESCRT-associated proteins (Pornillos et al., 2003). The ESCRT family of proteins comprise the cellular machinery able to carry out fusion and closure of membranes to create vesicles within the endosomal pathway as well as separation of cells during cell division (Carlton et al., 2007; Morita et al., 2007). This family of proteins make up the only cell machinery capable of severing the membrane ‘necks’ formed during vesicle formation or cell budding. Therefore, viruses like HIV-1 have exploited this apparatus, by recruiting ESCRT proteins by interaction with p6, in order to complete the closure of assembling virions, releasing them from the plasma membrane.

Maturation is the last step in the viral replication cycle and occurs under the direction of the virally encoded PR enzyme (Huang et al., 1995). Cleaving of the assembled viral polyproteins by PR forms a condensed mature particle (Gottlinger et al., 1991; Huang et al., 1995). The protease cascade occurs in an ordered fashion that is dictated by the relative affinities of the cleavage sites in the individual HIV-1 proteins for PR (Cimarelli et al., 2002). Mutation of these cleavage sites can result in the production of immature, non-infectious virions (Kohl et al., 1988; McQuade et al., 1990). Figure 1.9 summarises the full replication cycle of HIV-1.
Figure 1.9: The replication cycle of HIV-1

Figure 1.9. The HIV-1 replication cycle. Replication of HIV-1 is initiated with binding to the primary CD4 receptor (1) which exposes a conserved co-receptor binding site, facilitating the binding of CXCR4 or CCR5 chemokine co-receptors (2). Receptor binding exposes the gp41 fusion peptide that inserts into the cell membrane and rearrangement of gp41 helical domains mediates membrane fusion (3). After viral uncoating (4), the viral RNA genome is reverse-transcribed (5) into proviral DNA before association with cellular and viral proteins to form the pre-integration complex (PIC). Nuclear import translocates the PIC into the nucleus where the proviral DNA is integrated (6) into the host genome by the HIV-1 protein integrase. Transcription and translation (7) create both viral proteins and the full-length viral RNA genome required for assembly and budding of viral particles at the cell membrane surface (8). Viral replication is completed upon maturation (9) mediated by the proteolytic cleavage of viral proteins by HIV-1 protease.
1.7 HIV-1 proteins

1.7.1 HIV-1 Gag proteins

The HIV-1 Gag proteins (matrix, capsid and nucleocapsid) are encoded by a subgenomic mRNA and are translated as a single polyprotein, Gag (Pr55Gag). Gag proteins are sufficient for the formation of non-infectious virions, illustrating their role in virus morphology (Knipe et al., 2001). After virus assembly and budding at the plasma membrane, the Pr55Gag polyprotein is cleaved during viral maturation to yield the mature Gag proteins MA, CA, NC and p6 (Huang et al., 1995).

The HIV-1 CA was isolated as a 24 kilodaltons (kDa) protein immunologically reactive with infected patient sera (Casey et al., 1985). The CA (Figure 1.10A and B) can independently assemble into VLP’s and determines virion size (diameter) and morphology (Gelderblom et al., 1987; Ako-Adjei et al., 2005). This is mediated by Pr55Gag-Pr55Gag interactions via the multimerisation of CA, MA and NC domains during viral assembly (Momany et al., 1996; Ako-Adjei et al., 2005).

Monomeric CA contains an N’-terminus domain and C’terminus domain both of which contain multiple α-helices which form their quaternary structures (Momany et al., 1996; Ganser-Pornillos et al., 2004). Figure 1.10A and B illustrate the conformational structure of the two CA domains. Mutational analysis has identified that the N’-terminal region is required for proteolytic processing of the viral core on maturation, whereas the C’-terminal is crucial in dimerisation and Gag-Gag interactions during viral assembly (Momany et al., 1996).

Studies have demonstrated an early replication block on HIV-1 in monkey cells (Shibata et al., 1995; LaBonte et al., 2002). The CA protein has important functions during early replication events whereby cellular factors, such as cyclophilin A (CypA), interact with the viral core during the uncoating process (Luban et al., 1993; Franke et al., 1994; Thali et al., 1994; Braaten et al., 1996; Momany et al., 1996; Bosco et al., 2002; Owens et al., 2003). Therefore, it has been hypothesised that CA is involved in HIV-1 replication restriction in monkey cells, as species-specific cellular interacting
Figure 1.10. Mature HIV-1 Gag proteins.

Figure 1.10. Ribbon diagrams to illustrate the structure of the HIV-1 proteins (A) CA C-terminal dimerisation domain, (B) CA N-terminal dimerisation domain, (C) MA trimer and (D) NC. Diagram adapted from (Frankel et al., 1998).
factors, such as CypA, are required during this phase of the viral life cycle (Owens et al., 2003). This is due to CypA’s prolyl isomerise function that plays a role in the conformational changes that occurs in the CA protein during viral uncoating (Braaten et al., 1996; Bosco et al., 2002; Owens et al., 2003).

Other cellular factors cause species-specific restriction of retroviral replication. The TRIM5α proteins restrict HIV-1 replication by degrading viral complexes before reverse transcription. Expression of the rhesus macaque TRIM5α protein in human cells potently inhibits HIV-1 replication (Stremlau et al., 2004). Moreover, down-regulation of TRIM5α in rhesus macaque cells by RNAi increases HIV-1 infectivity (Stremlau et al., 2004). Human TRIM5α inhibits N-MLV infection in human cells and TRIM5α derived from New World monkeys can block infection by SIV strains (Hatziioannou et al., 2004; Keckesova et al., 2004; Perron et al., 2004; Yap et al., 2004). Therefore, TRIM5α is intricately involved in species-specific restriction of retroviral infectivity.

TRIM5α proteins are made up of four domains, a RING (really interesting new gene) domain, a B-box 2 domain, a coiled coil domain and a C’-terminal SPRY domain. TRIM5α multimerisation is required for its activity, which is mediated by the B-box 2 and coiled coil domains (Berthoux et al., 2005; Li et al., 2008). The C’-terminal SPRY domain creates the species specificity of TRIM5α through binding to the CA portion of Gag from different retroviruses (Nakayama et al., 2005; Perez-Caballero et al., 2005; Sebastian et al., 2005; Song et al., 2005; Yap et al., 2005; Stremlau et al., 2006). TRIM5α blocks viral replication by targeting retroviral RT complexes for proteosomic degradation through its RING domain, which possesses ubiquitin ligase activity (Wu et al., 1994). This is demonstrated by proteosome inhibitors which can reverse the action of TRIM5α (Wu et al., 1994). A derivative of TRIM5α, TRIM5-CypA, whereby the SPRY domain is replaced by CypA combines both CypA and TRIM in restriction of retroviral infection (Towers et al., 2003; Nisole et al., 2004; Sayah et al., 2004; Luban, 2007). TRIM5-CypA creates retroviral restriction by direct binding of TRIM-CypA to CA (Nisole et al., 2004; Sayah et al., 2004).
The mature MA protein (Figure 1.10C) is 17 kDa in size and folds into 5 α-helices, 4 of which compact to form an N’-terminal globular head (Hill et al., 1996). This structure is capped by a 3-stranded mixed β-sheet, containing a nuclear localisation signal thought to assist genome uncoating and entry into the host cell nucleus (Cannon et al., 1997; Hearps et al., 2007).

The native MA forms a trimer with a positively charged membrane binding surface (Massiah et al., 1994; Hill et al., 1996). The N’-terminus is myristylated enabling anchorage of the Pr55\textsuperscript{Gag} protein in the membrane during viral assembly, budding and release from the cell (Dorfman et al., 1994; Lee et al., 1997). The MA protein is involved in protein-protein and protein-membrane interactions during assembly at the plasma membrane (Facke et al., 1993; Hill et al., 1996; Lee et al., 1997). There is also evidence that MA aids RNA incorporation into new virions by binding to RNA molecules with sequence homology to the pol region of the HIV-1 genome (Purohit et al., 2001).

The mature NC protein (7kDa, Figure 1.10D) is produced during maturation, by proteolytic processing of the Gag polyprotein and forms a condensed helical conformation encapsulating the dimeric RNA genome in 2000 NC molecules (Darlix et al., 1995). The protein is rich in proline and basic amino acids and contains two CCHC or zinc finger motifs, brought together in a globular domain by a linker region, that binds Zn\textsuperscript{2+} with high affinity (South et al., 1993).

The NC can bind single-stranded DNA or RNA and protects the viral genome from nuclease attack (Darlix et al., 1995). Nucleic acid binding is dependant on basic residues within the protein, as well as the zinc finger motifs, and requires the dimerisation-encapsidation sequence (E-DLS) encoded in the viral genome (Morellet et al., 1992; Schmalzbauer et al., 1996; Mark-Danieli et al., 2005). NC has also been shown to possess annealing activity, promoting primer tRNA binding to the primer binding site (PBS) (Morellet et al., 1992).
Figure 1.11. The HIV-1 enzymes

A

B

C

Figure 1.11. Ribbon structure of the HIV-1 encoded enzymes. (A) RT dimer (B) Integrase (IN) domains (left to right): N-terminal domain dimer, catalytic dimer and DNA-binding domain dimer. (C) Protease (PR) dimer. Diagram adapted from (Frankel et al., 1998).
1.7.2 HIV-1 enzymes

The viral enzymes are encoded by the pol gene and translated into a single polyprotein in combination with the structural Gag proteins (Gag-Pol) (Ferris et al., 1990). The HIV-1 RT enzyme (Figure 1.11A) is essential during the viral replication cycle, coordinating the production of a double-stranded proviral DNA molecule, from the RNA genome template, prior to integration into the host cell chromosomes. Production of recombinant RT in Eshericia coli (E.coli) showed that protein synthesis of RT results in two protein species of 51 and 66 kDa in size, processed by the viral-encoded protease (the HIV-1 protease is discussed below) and contain identical N’-terminals (Farmerie et al., 1987; Jacobo-Molina et al., 1991; Gutierrez-Rivas et al., 2001).

The two proteins form the mature heterodimeric RT complex and both monomers are required for efficient enzymatic activity (Ferris et al., 1990; Jacobo-Molina et al., 1991). The p66 protein confers RNase H activity at the C’-terminus and polymerase activity at its N’-terminus, whereas the p51 protein catalyses RNA-dependent DNA polymerisation only (Ferris et al., 1990; Jacobo-Molina et al., 1991). The heterodimer hydrophobic interface is a tight interaction and once the mature p66:p51 protein is formed, the viral PR cannot catalyse cleavage of the larger p66 subunit (Jacobo-Molina et al., 1991).

Like polymerases from other organisms, RT contains subdomains (Figure 1.11A). Often compared to a hand, reflecting the overall shape of the molecule, these are named the palm, fingers, thumb and connection domains, (Huang et al., 1998; Gutierrez-Rivas et al., 2001). Conserved amino acids in the palm and finger domains of p66 along with α-helices from the thumb act as a clamp to position the template:primer complex relative to the active site (Huang et al., 1998). The active site of the polymerase is situated in the palm domain of p66 and contains catalytic asparagines residues (Asp 110, 185 and 186) (Huang et al., 1998). These residues are also important in forming the active site of the RNase H domain and require a magnesium cation for activity (Ferris et al., 1990; Jacobo-Molina et al., 1991; Huang et al., 1998). Analysis of the structure of an RT molecule with DNA:primer template and bound dNTP (deoxy-nucleotide triphosphate) molecule bound, led to the location of the nucleic acid primer terminus lying near the catalytic asparagines residues in the palm and the duplex
extending along the enzyme surface towards the RNase H domain of p66 (Huang et al., 1998). Binding of the nucleic acid in the enzyme palm, followed by dNTPs in the finger domain, induces a rate-limiting conformational change bringing the Mg\(^{2+}\)-ligated dNTP fingers closer to the active site. This leads to attachment of the dNTP molecule to the DNA:primer template 3’-OH phosphate (Huang et al., 1998). The finger domain can then move back to bind a new dNTP molecule ready to repeat the process and extend the DNA molecule (Huang et al., 1998).

The HIV-1 RT has a tendency to introduce mutations into the viral genome because of a lack of an editing mechanism. As a result, an average of 5 x 10^4 mutations per base pair per cycle are introduced into the HIV-1 genome (Roberts et al., 1988; Mansky et al., 1995). This allows HIV-1 to escape immune responses and antiviral agents through antigenic variation (Desrosiers et al., 2004). However, this value was estimated using a recombinant RT enzyme in vitro and estimates of single-round infection in vivo indicate a 6- to 15-fold lower rate of mutation (Mansky et al., 1995).

The viral RT enzyme is the first target for licenced anti-retroviral drugs which consist of nucleoside derivatives or non-nucleoside compounds (Furman et al., 1986; Jacobo-Molina et al., 1991). Reverse transcriptase inhibitors, like Zidovudine (AZT), are an example of the former and are incorporated into the DNA strand (Furman et al., 1986; Mitsuya et al., 1986). The 2’,3’-dideoxy configuration of this nucleoside derivative terminates DNA polymerisation and halts viral replication, as further phosphodiester linkage at the 3’ position of the sugar cannot occur (Rozenbaum et al., 1985). Resistance to nucleoside derivatives is common and occurs by mutation at inhibitor:protein contact sites, especially in the exposed disordered loops of the dNTP binding finger domains of RT (Huang et al., 1998).

Viral integration is catalysed by the viral IN (Figure 1.11B) enzyme which is a 32 kDa, ‘Y’ shaped protein made up of two homodimers forming a catalytic core at the interface (Chen et al., 2000; Gutierrez-Rivas et al., 2001; McKee et al., 2008). Evidence suggests HIV-1 IN functions in a tetramer formation, consisting of two dimers at each end of the viral DNA (Dyda et al., 1994; Heuer et al., 1998; Chen et al., 2000; McKee et al., 2008). Consisting of three distinct domains (Figure 1.11B), the IN monomer carries out both the 3’ end processing and transesterification reactions involved in
proviral integration into the host genome (Chen et al., 2000). The C’-terminal domain plays a role in DNA binding and orientation of the DNA for integration, as well as facilitating multimerisation of IN (Chen et al., 2000; Gutierrez-Rivas et al., 2001; McKee et al., 2008). The N’-terminal domain has conserved cysteine and histidine residues (forming a ‘CC-HH’ motif) that bind zinc ions and contribute to both protein multimerisation of IN and its catalytic function (Chen et al., 2000; McKee et al., 2008).

A strip of positive amino acid residues in each monomer comprises the active site in the catalytic core domain (Chen et al., 2000). A magnesium ion, which is chelated by essential amino acid residues (Asp64, Asp116 and Glu152) within the active site contributes to enzymatic function (Dyda et al., 1994; Esposito et al., 1998; Goldgur et al., 1999; Chen et al., 2000). Positive residues, including K159, Q148 and Y143 bind adenine in the genomic terminal 3’-CA signal and stabilises IN binding to viral DNA (Esposito et al., 1998; Chen et al., 2000). The presence of twelve bases on the 3’ viral LTR is essential for DNA binding to the large cleft formed by the SH3-like domain of the IN C’-terminus (Bushman et al., 1991; Esposito et al., 1998) and are important for DNA integration (Bushman et al., 1991).

Cellular factors are also involved in HIV-1 integration, one example being Lens epithelial-derived growth factor (LEDGF). The knock-down of cellular LEDGF, using RNA interference, reduces HIV-1 infectivity (Llano et al., 2006). Similarly, LEDGF knockout mouse embryonic fibroblasts exhibit reduced levels of HIV-1 infection (Emiliani et al., 2005; Marshall et al., 2007). The viral integrase recruits cellular proteins into the PIC which, in the case of LEDGF, tethers the complex to active genes and facilitates viral integration (Maertens et al., 2003; Emiliani et al., 2005; Llano et al., 2006).

The HIV-1 IN protein is an attractive target for the production of anti-retroviral drugs (Farnet et al., 1996). Initial studies showed that inhibitors can bind to residues in the enzyme active site that bind viral DNA (Lys156, Lys159, Glu148 and Tyr143), allowing interactions at sites that would represent substantial loss in enzyme catalytic activity and viral fitness (Goldgur et al., 1999). However, many compounds synthesised using purified IN showed reduced potency when tested against PIC complexes (Farnet et al., 1996). Therefore, assays utilising purified PIC complexes and
not purified IN protein are more useful in screening possible IN inhibitors (Farnet et al., 1996).

Despite these early set-backs, integrase inhibitors have reached phase III trials in the clinical setting. The integrase inhibitor Raltegravir inhibits viral replication by preventing proviral DNA-strand transfer (Markowitz et al., 2006; Kassahun et al., 2007). A phase III trial comparing Raltegravir against a placebo in patients with triple-class drug resistance has provided promising results (Steigbigel et al., 2008). At week 16 of treatment, 77.5% of patients in the Raltegravir arm had viral loads less than 400 copies/ml of blood, compared to 41.9% of patients in the control arm (Steigbigel et al., 2008). This trial indicated the promise for current and future application of integrase inhibitors into the clinic (Steigbigel et al., 2008; Markowitz et al., 2009).

Maturation is mediated by the HIV-1 protease (PR, Figure 1.11C), a 14kDa protein that is a member of the aspartic protease family of enzymes due to its structure and catalytic activity (Toh et al., 1985; Appelt et al., 1991). Comprised of 99 amino acids, it is synthesised as part of the Gag-Pol precursor polyprotein (Ishima et al., 2003). The mature protein is a homodimer with the active site at the interface of the multimer, with flexible flaps that loosely cover the active site (Toh et al., 1985; Appelt et al., 1991). The length of these flaps is important as the extension of the N’-terminal of the PR monomers at this flap region, leads to dimer dissociation and ablation of PR function (Ishima et al., 2003). The active site is formed by two conserved amino acid triads (Asp25, Thr26 and Gly27), one from each dimer, that form loop structures stabilised by hydrogen bonding (Navia et al., 1989; Wlodawer et al., 1989; Ishima et al., 2003; Tang et al., 2008). The active site is formed by two conserved amino acid triads (Asp25, Thr26 and Gly27), one from each dimer, that form loop structures stabilised by hydrogen bonding (Navia et al., 1989; Wlodawer et al., 1989; Geller et al., 1997; Ishima et al., 2003). Mutations in the active site key triad residues prevent polyprotein processing, producing immature, non-infectious particles (Miller et al., 1989).

Viral maturation occurs in a precise sequence of events that is initiated by the folding and dimerisation of PR whilst it is still associated as part of the Gag-Pol precursor (Ishima et al., 2003). This folding catalyses the hydrolysis of peptide bonds at its termini (Oroszlan et al., 1990). Intramolecular cleavage first at the N’-terminus p6/PR junction and then at the PR/Pol interface releases mature HIV-1 protease (Louis et al., 1994; Wondrak et al., 1996; Louis et al., 1999; Louis et al., 1999). Cleavage of PR is
the initial control step of maturation, as changes in this cleavage site produce an extended PR species with severe defects in Gag processing, leading to a loss in viral infectivity (Tessmer et al., 1998).

The viral PR is the first HIV-1 protein that has allowed the identification of new anti-retroviral therapies through structural inhibitor modelling. The binding of inhibitors, such as Saquinavir, induces large conformational changes in the viral protease active site that render the protein inactive (Furman et al., 1986). The success of protease inhibitors has led to their routine use in HAART triple therapy to reduce the emergence of drug resistance by targeting multiple HIV-1 replication pathways.

1.7.3 HIV-1 envelope glycoproteins

The role of the HIV-1 glycosylated envelope glycoproteins (Figure 1.6 shows the native envelope glycoprotein) is to mediate specific attachment to and entry of the virus into a target cell. The Env structure is made up of a trimer of non-covalently associated heterodimers consisting of a 120kDa surface unit (SU or gp120) and a 41kDa transmembrane protein (TM or gp41) (Kowalski et al., 1987; Hallenberger et al., 1992). The gp160 molecule is produced as a single chain protein precursor that is cleaved to form the gp120 and gp41 subunits by cellular proteases in the Golgi apparatus, for example furin and the two subunits associate to form the mature gp160 glycoprotein (Earl et al., 1990; Hallenberger et al., 1992).

Cyroelectron microscopic tomography has shown that SIV and HIV envelope glycoproteins exist in ‘spike’ trimer arrangements with globular gp120 subunits in a right-handed propeller orientation, over a compact ‘stem’-like gp41 TM protein (Zanetti et al., 2006; Liu et al., 2008). Visualisation of glycoproteins in this way has allowed the calculation of trimeric complexes, 7-14 (+ or – 7) spikes per virion, with no evidence of periodic glycoprotein spacing or glycoprotein movement within the viral envelope (Zanetti et al., 2006; Liu et al., 2008).

The surface unit, gp120, is composed of 5 amino acid sequence conserved regions (C1-5) interspersed with 5 variable regions (V1-5), the first 4 of which create surface exposed loops containing disulphide bonding at their bases (Kwong et al., 1998). The core of gp120 folds to create inner and outer domains connected by a β-sheet region,
Figure 1.12. Domain structure of HIV-1 gp120. The surface unit, gp120, is composed of 5 amino acid sequence conserved regions (C1-5) interspersed with 5 variable regions (V1-5), the first 4 of which create surface exposed loops containing disulphide bonding at their bases. The core of gp120 folds to create inner (red) and outer (yellow) domains connected by a β-sheet region termed the bridging sheet (blue). The inner domain makes contacts with the other heterodimers and is located within the envelope spike. The outer domain is exposed on the outside of the protein and is heavily glycosylated. Diagram adapted from HIV-1 database at www.hiv.lanl.gov.
termed the bridging sheet (Figure 1.12) (Kwong et al., 1998; Zanetti et al., 2006). The inner domain makes contacts with the other heterodimers and is located within the envelope spike and is relatively conserved. The inner domain possesses electroneutral surfaces, combined with a lack of glycosylation that facilitates trimer packing (Kwong et al., 1998). The inner domain also contains both the C'- and N’-termini which are orientated close to the viral membrane (Kwong et al., 1998; Zanetti et al., 2006). The exposed outer domain contains significant variable regions and is heavily glycosylated, the carbohydrate residues accounting for 40%-50% of the molecular weight (Poumbourios et al., 1995; Kwong et al., 1998; Zanetti et al., 2006).

The interactions between gp120 and gp41 enabling heterodimer and Env trimer formation are still elusive, but numerous studies have implicated multiple regions in gp160 that may play a role in this interaction (Helseth et al., 1991; Poumbourios et al., 1995; Wyatt et al., 1998; Kao et al., 2001; York et al., 2004; Kim et al., 2008). Mutations in gp41, particularly in the N’-terminal ectodomain, can lead to an increase in gp120 shedding as a result of reduction in the association between the two glycoprotein subunits (Helseth et al., 1991; Poumbourios et al., 1995; Wyatt et al., 1998; Kao et al., 2001; York et al., 2004; Kim et al., 2008). Conserved residues in the ectodomain of gp41 have also been linked to oligomerisation of the Env spike complex (Poumbourios et al., 1995; York et al., 2004). Insertion of amino acids into the N’- or C’-termini of gp120 resulted in dissociation of Env subunits, both of which are highly conserved regions important in forming the gp120:gp41 interface (Helseth et al., 1991; Poumbourios et al., 1995; Wyatt et al., 1998; Kim et al., 2008). Conserved hydrophobic stretches in the C1, C3, C4, and C5 regions of gp120 are also implicated in the SU:TM interaction (Cao et al., 1993). Changes in amino acid sequence at the glycoprotein subunit interface may be due to the interruption of contacting amino acids or a change in protein conformation that hinders subunit association (Helseth et al., 1991).

Key experiments involving the production of a gp140 glycoprotein molecule engineered with a disulphide bond between subunits has further allowed the identification of regions involved in the gp120:gp41 interaction along with the proteolytic cleavage of gp160 (Sanders et al., 2000). Deletion of the variable regions V1-V3 inhibited the formation of disulphide bonded gp140 by preventing proteolytic processing of the
protein precursor (Sanders et al., 2000). However, deletion of each variable region individually showed no change in gp140 formation (Sanders et al., 2000). Moreover, deletion of the V3 domain in the wildtype non-covalently associated gp140, prevented proteolytic separation of the two proteins, implicating V3 in protein cleavage (Sanders et al., 2000). Therefore, many protein domains of both gp120 and gp41 are important in heterodimer interactions.

The CD4 binding site depression, present at the interface between the outer domain, inner domain and the bridging sheet, is highly conserved and devoid of carbohydrate residues (Kwong et al., 1998). The C4 region of gp120 is important in the interaction of the virus with the primary receptor, CD4, as deletions in this region impair receptor binding (Moore et al., 1993). Monoclonal antibodies directed against the C4 region have also been shown to block CD4 binding (Helseth et al., 1991). The point of gp120-CD4 contact has electrostatic complementarity (Kwong et al., 1998). The CD4 binding depression contains two cavities, one shallow and one deep (Kwong et al., 1998; Wyatt et al., 1998; Wyatt et al., 1998). The deep cavity contains variable residues and binds water molecules, whereas the buried shallow cavity is hydrophobic and directly involved in binding CD4 (Kwong et al., 1998; Wyatt et al., 1998; Wyatt et al., 1998). A large surface area of 24 amino acids of CD4 and 26 amino acids of gp120 allow receptor interaction, co-ordinated by van der Waals interactions and hydrogen bonding (Kwong et al., 1998).

On CD4 binding, conformational changes within gp120 cause the V1-V3 loop structures to move, exposing the co-receptor binding site (Moore et al., 1993; Wyatt et al., 1998). It has been hypothesised that they represent the primordial obligate receptor for retroviruses, due to the fact that other retroviruses, such as Feline Immunodeficiency Virus (FIV) utilise chemokine receptors as the primary receptors, while the evolution of CD4 usage results from host immune evasion of the conserved glycoprotein co-receptor sequences (Wyatt et al., 1998). The V3 loop is particularly important in determining co-receptor usage and cell tropism. This was demonstrated by the interchange of the V3 region from a macrophage-tropic isolate to HIV-1IB, conferring macrophage tropism to this CXCR4-restricted isolate (Hwang et al., 1991; Cann et al., 1992).
The gp41 subunit of gp160 is responsible for the molecular events involved in membrane fusion. It has three distinct domains, including an ectodomain exposed on the outside of the virion which is immunogenic, the transmembrane domain spanning the viral envelope and a long 150 amino acid cytoplasmic tail (Kim et al., 2008). The protein is made up of two heptad repeats (HR) designated HR1 and HR2. The prefusogenic conformation of gp41 is still the subject of investigation, but is thought to consist of a hetero-trimeric helix conformation, with heptad repeats binding to similar regions in the other monomers (for example HR1 bound to HR1 and HR2 bound to HR2) (Kwong et al., 1998; Kim et al., 2008).

More is known about the fusion active conformation of gp41 as there is a strong tendency for gp41 to exist in this state (Kim et al., 2008). The conformation of gp160 pre-receptor binding sequesters vital regions of gp41 that readily form a six helical bundle composed of HR1 and HR2 regions of individual monomers interacting to create hairpin structures. Trimers of these hairpin structures create the fusion bundle (Kim et al., 2008) which facilitates viral fusion (Kwong et al., 1998; Wyatt et al., 1998).

The importance of these hairpin structures in HIV-1 fusion is shown by the effectiveness of gp41 peptide mimics. Short peptides are able to bind to gp41 and prevent bundle formation. Resistance to these peptides by mutational escape is limited, due to the need for conservation of amino acid sequence in this region of gp41 (Moore et al., 1993; Wyatt et al., 1998).

1.7.4 HIV-1 accessory proteins

The HIV-1 virus encodes six accessory proteins (Vif, Vpu, Vpr, Nef, Tat, and Rev), resulting from translation of spliced mRNA transcripts. These proteins are important during multiple stages of the replication cycle.

The Viral Infectivity Factor (Vif) is essential for in vivo survival of HIV-1, allowing replication in lymphocytes and macrophages (Lv et al., 2007). Although no crystal structure exists for the 23kDa HIV-1 Vif protein, two functionally crucial domains have been identified by mutational analysis, the N’-terminal and C’-terminal domains. The N’-terminal domain irreversibly binds to APOBEC3G, a cellular nucleic acid editing
enzyme which would otherwise be packaged into the virion and cause mutation of newly synthesised DNA during reverse transcription (Mangeat et al., 2003; Zhang et al., 2003; Cancio et al., 2004; Lv et al., 2007). The C’-terminal domain of Vif is involved in APOBEC3G degradation, as it contains a conserved motif (amino acid sequence SLQ(Y/F)ΦΦΦΦ) that interacts with the cellular Elongin complex, targeting APOBEC3G to the ubiquitin-proteasome pathway for degradation (Marin et al., 2003; Sheehy et al., 2003; Lv et al., 2007).

The Vpr protein is encapsidated in the virion particle and is present at a ratio of 1:7 Vpr:Gag by direct interaction with the C’-terminal of the p6 (Gag) protein during viral assembly (Paxton et al., 1993; Bachand et al., 1999; Muller et al., 2000). It is a small, 14kDa basic protein which is made up of 3 α-helices around a hydrophobic core surrounded by flexible N’ and C’-terminal domains (Morellet et al., 2003). The protein interacts with many cellular partners possessing WxxF amino acid motifs, for which the function in viral replication is still unknown (Bukrinsky et al., 1999; Le Rouzic et al., 2005). Therefore, many other functions of Vpr have been suggested but not yet defined. Vpr shares sequence similarity with the Vpx SIV/HIV-2 protein and the lack of Vpx in HIV-1 illustrates the evolution of Vpr function to incorporate the function of Vpx (Tristem et al., 1992; Sharp et al., 1996).

The Vpr protein is dispensible for replication in T cell lines, but critical for efficient in vivo HIV-1 replication (Bukrinsky et al., 1999). Deleted Vpr mutants (for example SIVmacΔVpr) are responsible for a much reduced viral burden and longer progression time to AIDS (Gibbs et al., 1995; Hoch et al., 1995). High frequencies of mutations within Vpr (Arg77 in particular) are also found in Long Term Non-progressors (LTNPs), demonstrating a role for Vpr in HIV-1 virulence (Somasundaran et al., 2002). The first Vpr function identified was in nuclear import of the HIV-1 pre-integration complex (PIC) into the cell nucleus. Targeting of the PIC to the nuclear envelope is facilitated by Vpr which resembles importin-β, a nuclear import receptor (Vodicka et al., 1998). This function of Vpr is essential for viral replication in non-dividing cells, like macrophages, where access to the nucleus is selective (Heinzinger et al., 1994; Vodicka et al., 1998; Sherman et al., 2001).

1 – Φ represents a hydrophobic amino acid
Vpr can cause cell cycle arrest in cells from a number of species, including human, simian and yeast cells, indicating conservation of the mitotic pathway across diverse microorganisms. Vpr prevents the dephosphorylation of p34\textsuperscript{cdc2} (a cyclin kinase) in turn rendering inactive its cell cycle partner, cyclin B, preventing the G\textsubscript{2}-M transition (He \textit{et al.}, 1995; Re \textit{et al.}, 1995). Arrest of infected cells in G\textsubscript{2} phase of the cycle is thought to increase HIV-1 replication as the HIV LTR is most active during G\textsubscript{2} (Le Rouzic \textit{et al.}, 2005).

The accessory protein, Tat, is a 14kDa protein produced early in infection and is a positive factor involved in the expression of viral transcripts from the promoter located in the 5’ LTR (Muller \textit{et al.}, 1990). The importance of Tat is reflected in its conservation in all lentiviruses, and that HIV-2 can be transactivated by the HIV-1 Tat, despite their genetic diversity (Weeks \textit{et al.}, 1990). The Tat protein is critically required for viral replication and interacts with the TAR region of the genome, which is a stem-loop structure located downstream to the transcription initiation site and, therefore, is cis-acting to the viral promoter (Peterlin \textit{et al.}, 1986). Both the RNA sequence and secondary structure of the TAR element are important for Tat binding and transcriptional trans-activation, as mutation of important bases and the alteration of the protein secondary structure within TAR ablates Tat function (Weeks \textit{et al.}, 1990).

The enhancement of viral transcript expression by Tat specifically increases the number of full-length viral mRNAs by promoting transcription elongation (Arya \textit{et al.}, 1985; Miller \textit{et al.}, 2000). Transfection of the LTR of HIV-1 linked to the CAT gene into HIV-1 infected or uninfected cells illustrates that while viral transcription can be initiated efficiently by the LTR alone, Tat increased CAT expression 1,100-fold (Sodroski \textit{et al.}, 1985). The Tat protein has also been linked to enhancement of translation events, as an increase in viral mRNA cannot account for the increase in viral protein alone (Wright \textit{et al.}, 1986).

The Tat protein directs viral transcription by interacting with many cellular proteins including transcription factors like TFIID and CREB (cAMP response element binding)-binding protein (CBP) (Liu \textit{et al.}, 2002). The best characterised of these interactors is human cyclin T which forms a complex with Tat and a cyclin-dependant kinase (CDK9), a component of the transcription elongation factor b (P-TEFb) (Garber
et al., 1998; Wei et al., 1998). This complex is recruited to the TAR element where CDK9 phosphorylates RNA polymerase II to initiate transcription (Garber et al., 1998; Wei et al., 1998).

The N’-terminus of Tat contains 4 potential cysteine-rich zinc-binding motifs that are thought to be important in linking Tat-associated mRNAs to the nuclear matrix. This allows HIV-1 transcription and mRNA transcript processing to proceed simultaneously (Garcia et al., 1988; Muller et al., 1990; Baier-Bitterlich et al., 1998). The C’-terminus basic region consists of mainly arginine and lysine residues important for TAR binding (Garcia et al., 1988; Baier-Bitterlich et al., 1998). In addition to these structures, the Tat protein forms two cavities on the surface facilitating protein:protein interaction.

The Vpu protein (Viral protein u, 16kDa), unique to HIV-1 (Chen et al., 1993), is translated from bicistronic mRNA (refer to Figure 1.3) together with the HIV-1 Env protein precursor (Strebel et al., 1988; Schwartz et al., 1990). It is highly conserved among HIV-1 isolates, indicating its importance during viral replication (Strebel et al., 1988). The Vpu protein is phosphorylated and has a hydrophobic 27 amino acid stretch characteristic of an integral membrane protein. Although Vpu is associated with the cell membrane, the protein is not incorporated into virions (Strebel et al., 1988; Strebel et al., 1989; Maldarelli et al., 1993).

The Vpu accessory protein is vital for virion release after assembly, as mutation of Vpu significantly decreases particle budding from the plasma membrane with alternative assembly and release at internal membranes (Strebel et al., 1988; Strebel et al., 1989; Terwilliger et al., 1989; Klimkait et al., 1990; Neil et al., 2008). Vpu acts to antagonise host restriction activity that impedes particle release in an independent mechanism to the ESCRT pathway. Neil et al. (2006) demonstrated that p6 mutants maintained the attachment between the HIV-1 envelope and the cell membrane for incomplete immature virions. Alternatively, the absence of Vpu in HIV-1 created fully formed and mature virions unable to detach from the cell membrane, which could be released by protease treatment, indicating a very late block in viral release (Neil et al., 2006). Therefore, these two mechanisms restricting viral release from the cell membrane were independent of each other.
The tetherin protein (previously named BST-2, CD 317 or HM1.24) blocks the release of retroviruses, filoviruses and arenaviruses (Jouvenet et al., 2009; Kaletsky et al., 2009; Sakuma et al., 2009). Tetherin cross-links cellular and viral membranes in a protease sensitive mechanism preventing the release of progeny virions (Neil et al., 2006; Neil et al., 2008). Vpu acts as an antagonist to tetherin restriction of viral particle release. Vpu has been shown to colocalise with tetherin in intracellular compartments such as the Golgi preventing association of tetherin with virions at the cell membrane, thereby downregulating the amount of tetherin at the plasma membrane (Neil et al., 2008; Jouvenet et al., 2009). Vpu is also thought to downregulate the total levels of tetherin in cells reducing its effect of viral budding (Van Damme et al., 2008). However, the precise mechanisms of tetherin action and Vpu antagonism of tetherin remain unclear.

The HIV-1 Regulator of protein expression (Rev) protein is 18kDa in size and composed of two discrete domains. The N’-terminal arginine-rich domain comprises both the nuclear localisation signal (NLS) and an RNA binding motif, flanked by multimerisation sequences (Perkins et al., 1989; Zapp et al., 1989; Olsen et al., 1990; Bohnlein et al., 1991; Malim et al., 1991; Zapp et al., 1991). The C’-terminal leucine-rich domain contains the nuclear export signal (NES) (Fischer et al., 1995; Meyer et al., 1996). Rev binds to the Rev response element (RRE) present within the env intron (Daly et al., 1989; Zapp et al., 1989). The RRE forms a secondary RNA structure forming a single high affinity Rev binding site in the major groove of the RNA made up of two non-Watson and Crick base pairs (G-G) (Malim et al., 1989; Charpentier et al., 1997).

Rev functions in the stabilisation and export of unspliced viral mRNA from the cell nucleus that would otherwise be retained within the nucleus until spliced or degraded (Felber et al., 1989). The Rev protein is fundamental to the nuclear export of full-length (9kB) and 4kB mRNA transcripts, but not smaller mRNA’s (2kB) which can pass into the cytoplasm unaided. Therefore, Rev is responsible for the biphasic gene expression typical of HIV-1 (Kim et al., 1989; Malim et al., 1989; Pomerantz et al., 1990). Smaller, 2kB mRNA transcripts accumulate in the cytoplasm and encode Rev itself, amongst other proteins, and is imported into the nucleus by interacting with the import receptor, importin-β (Henderson et al., 1997). Rev can then aid the export of
larger mRNA products encoding Gag, Pol and Env and is directly responsible for the initiation of viral progeny production (Malim et al., 1989).

The functions of the Negative regulatory factor (Nef, 27 kDa) accessory protein are highly debated. Structures identified to be important for its function include a myristylation signal involved in association of Nef with cell membranes, an N’-terminal α-helix, a central SH3-binding proline based repeat and a cluster of acidic amino acids also in the N’-terminal (Piguet et al., 2000). Nef dimerisation is also critical for protein function (Geyer et al., 2001).

The presence of Nef during replication significantly enhances virion infectivity. Nef supports HIV-1 replication, particularly in PMBCs, by activating the cellular signalling enzyme, Pak kinase (also referred to as Nak kinase, Nef-associated kinase), which in turn stimulates the activation of these cells, resulting in increased HIV-1 infectivity and viral production (Saksela et al., 1995; Sawai et al., 1995; Fackler et al., 2000).

Nef is responsible for the down-regulation of cellular proteins on HIV-1 infection of a cell. Two discontinuous regions of Nef (amino acids 66-144 and 175-186) which are conserved and juxtaposed at a single surface in the three-dimensional protein structure, are essential for downregulating CD4 from the cell surface, preventing superinfection (Aiken et al., 1994; Goldsmith et al., 1995; Aiken et al., 1996; Hua et al., 1997; Geyer et al., 2001). Nef also downregulates class I major histocompatibility complex (MHC I) expression at the cell surface, decreasing the recognition and killing of HIV-1 infected cells by cytotoxic T cells (CD8+ T cells) (Piguet et al., 2000; Geyer et al., 2001).

1.8 Lipid rafts and the viral envelope

Cellular membranes are made up of a lipid bilayer containing phospholipids like phosphatidylcholine (PC) or phosphatidylinositol (PI), glycosphingolipids (GSLs) such as sphingomyelin (SM) and cholesterol (Figure 1.13) (Fantini et al., 2002). Membrane proteins are integrated across the lipid bilayer or associate with each leaflet internally or externally. Despite the universal acceptance of the fluid-mosaic lipid bilayer model it was later discovered that the composition of the cell membrane is not uniform. Small
Figure 1.13. Lipid structure and organisation of biological membranes. A – Lipid molecules in the plasma membranes comprise glycerophospholipids (GPL’s), such as phosphatidylcholine (PC), glycosphingolipids (GSL’s) like sphingomyelin (SM) or GalCer and sterols, in the case of mammals, cholesterol. All membrane lipids contain fatty acid chains (black), polyunsaturated or saturated in the case of GPL’s and GSL’s respectively, and ranging in size. Membrane lipids possess either a glycerol (GPL’s) or sphingosine (GSL’s) backbone (green). Glycerophospholipids contain a phosphate head group (red) and can be decorated with alcohol moieties (blue). Glycosphingolipids on the other hand may, like GPL’s, possess phosphate (red) and alcohol (blue) headgroups or can be glycosylated with sugar residues (red in case of GalCer). B – The biological membrane bilayer is formed due to the amphipathic character of its lipid. Hydrophilic headgroups are exposed to the external aqueous environment while the hydrophobic tails are buried within the lipid bilayer. Most GPL’s are roughly cylindrical in structure and therefore form the liquid-crystalline phase (Lc) or “fluid-state” of the membrane which allows lateral diffusion of membrane molecules and flexibility and in almost devoid of cholesterol. With larger headgroups, GSL’s form a closely packed liquid-ordered phase (Lo) or lipid raft microdomains enriched with cholesterol. Figure adapted from (Fantini et al., 2002).
areas of the cell membrane could be extracted in detergent at low temperatures (on ice) and these areas were termed lipid rafts (or detergent resistant membranes) due to their unique composition compared to the normal plasma membrane (Manes et al., 2000; Fantini et al., 2002). Lipid rafts are mainly composed of cholesterol and sphingolipids and are less fluid than non-raft areas due to the tight packing of the lipids (Manes et al., 2000; Fantini et al., 2002; Munro, 2003). These small (50-70nm in size) areas of the membrane are associated with cellular signalling, allowing the congregation of membrane proteins into discrete domains (Manes et al., 2000; Simons et al., 2002; Rajendran et al., 2005).

The HIV-1 virus has a lipid envelope which is different from the cellular plasma membrane (Aloia et al., 1993). Clues pointing to the importance of lipid rafts with respect to HIV-1 came about before the recognition that these distinct membrane microdomains existed. The levels of PC and PI are reduced by at least 50% whereas cholesterol and GSL’s are present in up to 3-fold higher concentrations than that found in cellular membranes (Aloia et al., 1993). The cholesterol to phospholipid molar ratio of the cell membrane is 0.39, whereas that of different HIV-1 isolates can vary from 0.9 to 1.2, illustrating increased cholesterol levels (Brugger et al., 2006). Hence, the viral envelope is rich in cholesterol, causing it to be thicker and more rigid than the cell plasma membrane (Munro, 2003). This high level of cholesterol is due to the fact that HIV-1 buds selectively from lipid rafts areas of the membrane and, therefore, is mostly lipid raft-like in composition (Nguyen et al., 2000). The importance of lipid raft biology with respect to HIV-1 is well recognised and is involved in multiple parts of the replication cycle. Immature Gag protein multimerisation is targeted to lipid raft structures due to its myristylated N’-terminal (Ono et al., 2007). Therefore, localisation of HIV-1 proteins into lipid rafts in the cell membrane aids efficient virion assembly. The envelope gp41 protein also contains a cholesterol binding motif at amino acid residues 678-683 (LWYIK) promoting incorporation of Env into lipid rafts and progeny virions (Vincent et al., 2002). Therefore, evidence suggests that lipid rafts are important in viral budding and release from the infected cell by recruiting and localising important viral proteins to distinct, concentrated areas of the cell plasma membrane.

Evidence identifying lipid rafts as the site of viral budding includes the exclusion or inclusion of cellular proteins in the HIV-1 envelope. For example, rafts act to exclude
proteins, like CD45, that are large (CD45 has a 45nm exterior domain and a 700 amino acid cytoplasmic tail) which may sterically hinder virion formation and binding to target cells (Nguyen et al., 2000). Raft protein markers, such as GM1, Thy-1, GPI-linked proteins and CD59 are present in the viral envelope and co-localise with HIV-1 proteins and lipid rafts in infected cells (Nguyen et al., 2000).

Like the elevated cholesterol levels found in HIV-1 envelopes, infected-cell membrane cholesterol composition is greater than in uninfected cells (Aloia et al., 1993). Microarray analysis carried out by van t’ Wout et al (2005) revealed that HIV-1 infection increased the expression of cholesterol-related genes in both cell lines and primary CD4+ T cells. These changes were only observed in the presence of functional Nef and, therefore, Nef plays a role in cellular cholesterol production during HIV-1 replication (Zheng et al., 2003; van ’t Wout et al., 2005; Lai, 2009). Nef binds to and recruits cholesterol to the membrane by binding to cholesterol via its C’terminus, mutation of which ablates this property, compromising HIV-1 infectivity and target cell fusion ability (Zheng et al., 2003). Nef also promotes cholesterol accumulation within the infected cell by directly inhibiting ABCA1 (ATP-binding cassette transporter A1) control of cholesterol efflux (Mujawar et al., 2006).

Cholesterol and lipid rafts have been implicated in HIV-1 virus-cell and cell-to-cell membrane fusion. Depletion of viral envelope cholesterol, by up to 80%, significantly reduces HIV-1 infectivity, while maintaining protein composition and viral structure (Campbell et al., 2002; Guyader et al., 2002; Graham et al., 2003). At extreme levels of cholesterol depletion (80-100%) permeabilisation of virions with a loss of internal proteins has been demonstrated (Graham et al., 2003).

The reduction in HIV-1 infectivity by envelope cholesterol removal inhibits viral fusion and internalisation (Manes et al., 2000; Guyader et al., 2002) which can be reversed by cholesterol replenishment (Manes et al., 2000). Viral binding to target cells through gp120-CD4 interactions, however, is not impeded by cholesterol depletion, as receptor binding at 4°C (a temperature at which this can occur but fusion is prevented) has been observed (Manes et al., 2000; Guyader et al., 2002). With temperature increase, the
lack of cholesterol inhibits the subsequent post-binding fusion steps of both X4 and R5 HIV-1 strains (Manes et al., 2000; Guyader et al., 2002). This inability to fuse with the cell results from prevention of the clustering of lipid raft microdomains that facilitate co-receptor interaction and, hence, membrane fusion (Manes et al., 2000).

Not only is cholesterol required in the viral envelope for fusion and cell entry (Manes et al., 2000; Campbell et al., 2002; Guyader et al., 2002; Graham et al., 2003), but the cholesterol content of the target cell membrane is of pivotal importance, as cholesterol depletion of uninfected cells inhibits free virus fusion (Liao et al., 2001; Liao et al., 2003). Viard et al (2002) showed that cholesterol is important in primary cell (T cell)-virus fusion, whereas the same was not true for T-cell lines. Multiple CCR5 molecules (up to 6 molecules) are required to produce a fusion event in activated lymphocytes and, therefore, as only approximately 1,000 co-receptor molecules are present on the 500µm² cell surface of T lymphocytes, the average distance between co-receptors is 0.7µm (Viard et al., 2002). These dimensions are unlikely to generate receptor clusters large enough for HIV-1 fusion (Viard et al., 2002) and, hence, lipid rafts are vital in forming receptor clusters of adequate spatial orientation, approximately 10nm apart (Popik et al., 2002; Yi et al., 2006). Therefore, it was hypothesised that lipid rafts facilitate formation of receptor recruitment sites in circumstances where cellular receptor levels were low, as in the case of primary T lymphocytes (Viard et al., 2002).

Lipid rafts play an important role in receptor clustering and recruitment during viral fusion, but specific interactions between HIV-1 receptors and lipid rafts in cell membranes is not required (Manes et al., 2000; Guyader et al., 2002). Cholesterol, however, is a structural requirement in the viral envelope for infectivity and fusion ability. There are exceptions to these rules, particularly where HIV-1 infection occurs in non-CD4⁺ cells. For example, Argyis et al (2003) reported that membrane cholesterol depletion of human brain microvascular endothelial cells (HBMVEC’s) did not ablate the infectivity and fusion potential of HIV-1, as cell entry was mediated by cell-associated chondroitin sulphate proteoglycans as alternative receptors (Argyris et al., 2003).
Membrane GSL reduction also has similar effects on free virus and infected cell-cell fusion as cholesterol removal. Reduction of GSL membrane concentrations using biosynthesis inhibitors (1-Phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP) or L-cycloserine) can not only reduce HIV-1 infectivity by blocking viral fusion, but is particularly critical when cell receptor levels are low (Rawat et al., 2005; Rawat et al., 2006). The influence of membrane GSL’s, however, is limited to co-receptor interactions, as GSL depletion does not inhibit fusion of CD4-independent HIV-1 strains (Ablan et al., 2006). Cellular cholesterol, in contrast, is required for both CD4 and co-receptor engagement during cell entry, as depleting membrane cholesterol inhibits the fusion of these same CD4-independent HIV-1 strain (Ablan et al., 2006).

The spread of HIV-1 can occur directly from T cell to T cell using supramolecular structures termed virological synapses (McDonald et al., 2003; Jolly et al., 2004). This mode of infection is productive and more rapid than free virus infection and is thought to contribute to HIV-1 spread in vivo (Sato et al., 1992). Cholesterol is required in this instance as membrane depletion of cholesterol disrupts Gag-Env interactions that are polarised within lipid rafts at the virological synapse (Jolly et al., 2005).

During primary infection, HIV-1 crosses the mucosal epithelia via the process of transcytosis (Bomsel, 1997). Transcytosis is mediated by galactosylceramide (GalCer), a glycosphingolipid commonly found in lipid rafts, at the apical surface of epithelial cells (Simons et al., 1988; Yahi et al., 1992) implicating lipid rafts in the initial steps of HIV-1 infection (Alfsen et al., 2001).

1.9 HIV-1 and the immune system

A significant problem in HIV vaccine development is that the true nature of the immunity required for controlling infection is still unclear. The contribution of different immune components and which of these are dominant during HIV-1 infection is complex and will now be discussed.
1.9.1 HIV-1 humoral responses

A neutralising antibody response to HIV-1 can appear within weeks of infection and may contribute to the control of primary viraemia (Pellegrin et al., 1996; Binley et al., 1997; Pilgrim et al., 1997; Aasa-Chapman et al., 2004). The proteins of HIV-1 are as immunogenic as comparable proteins of other viruses (Sattentau et al., 1999) and, therefore, antibodies are commonly produced in vivo (Pellegrin et al., 1996; Binley et al., 1997; Pilgrim et al., 1997; Aasa-Chapman et al., 2004). Despite this, the majority of antibodies elicited by the glycoproteins in vivo are non-neutralising and have little efficacy in controlling infection (Busch et al., 1995). Moreover, to ensure antibody responses can combat HIV-1 infection, all functional trimers on each virion would need to be bound by antibody and neutralised, as 1 viral spike may be sufficient for viral entry (Klasse et al., 2002; Yang et al., 2005; Yang et al., 2005). Therefore, a neutralising antibody response that can control or even prevent infection must be a potent one.

However, the surface glycoproteins of HIV-1, gp120 and gp41, are highly evolved to protect conserved receptor binding surfaces from antibody attack (Wyatt et al., 1998). High amounts of glycosylation, recessed motifs and high variability in protein sequence prevents antibody binding to gp160 (Poignard et al., 1996). Those binding surfaces that are well conserved (particularly gp41) are only exposed for a short time, reducing the possibility for antibody binding during viral attachment (Sattentau et al., 1999; Zwick et al., 2001).

Protection against HIV-1 infection by humoral immunity is feasible, but investigation of passive antibody transfer has provided mixed results (Prince et al., 1988; Putkonen et al., 1991; Emini et al., 1992; Almond et al., 1997; Parren et al., 2001; Veazey et al., 2003). In one study, intravenous infusion of purified anti-HIV-1_IIIB antibodies into chimpanzees produced a circulating neutralising antibody titre of 1 in 320, which provided protection against challenge with HIV-1_IIIB (75 chimpanzee infectious doses, ID_{50}) 24 hours later (Emini et al., 1992). This study showed that protection against infection was feasible, if circulating antibodies are present.
Despite these positive results, earlier studies have questioned the feasibility of protection from infection by antibody transfer. For example, passive immunisation of chimpanzees with concentrated human antisera failed to protect chimpanzees from infection after receipt of a HIV-1\text{IIIb} challenge (400 TCID\textsubscript{50} or 100 ID\textsubscript{50} per chimp) 24 hours post-infusion (Prince \textit{et al.}, 1988). Putkonen \textit{et al.} (1991) demonstrated that passive immunisation of cynomolgous monkeys (Macaca fascicularis) using SIV\textsubscript{sm} heat-treated antiserum protected only 3 of 4 animals challenged with 10-100 MID\textsubscript{50} (monkey infectious doses at 50\%) of SIV\textsubscript{sm} 6 hours post-infusion. Although complete protection for all animals was not achieved, in comparison with control animals, all of which became infected, passive immunisation significantly reduced the risk of infection (Putkonen \textit{et al.}, 1991).

The aforementioned studies have failed to define protection against HIV-1 infection by passive immunisation (Prince \textit{et al.}, 1988; Putkonen \textit{et al.}, 1991; Emini \textit{et al.}, 1992; Almond \textit{et al.}, 1997; Parren \textit{et al.}, 2001; Veazey \textit{et al.}, 2003). Saying this, it is difficult to compare HIV-1 infection in humans by analysing infection of different animal models utilising varying challenge doses. Despite the indications of these passive immunisations studies, assessment of infection as close to the natural process as possible will be more insightful.

For example, investigating protection in animal models by challenge with the actual infectious dose encountered on sexual exposure to HIV-1 would indicate the level of circulating antibody required for protection against natural infection. Similarly, titration of the antibodies used in passive immunisation would also indicate the titres required for protection against challenge in animal models. A study by Parren \textit{et al.} (2001) has demonstrated that administration of the b12 MAb intravenously into macaques can protect against vaginal challenge with an R5 SHIV\textsubscript{162P4} virus (300 TCID\textsubscript{50}) 6 hours post-infusion. This study investigated the level of circulating antibody required to provide protection from infection and demonstrated that 25mg of b12 per kilogram (kg) of body weight protected 4 out of 4 animals later challenged giving sterilising immunity (Parren \textit{et al.}, 2001). Animals were administered 5mg/kg of b12 had a 50\% rate of infection post-challenge, illustrating that the threshold of circulating antibody required to give sterile protection was above this level of passive immunisation, however, plasma viraemia was delayed and reduced in this group of animals (Parren \textit{et al.}, 2001). A third
group of macaques were given just 1mg/kg of b12 and all animals became infected (Parren et al., 2001).

Therefore, these investigations indicated that intravenous infusion of a potent neutralising antibody could protect against vaginal challenge. A 1 in 400 circulating titre of neutralising antibody was subsequently calculated to be required for sterile protection, with a 1 in 100 titre effecting lowered viraemia (Parren et al., 2001). This model also utilised an R5 isolate which closely mimics natural infection, indicating the real possibility of preventing infection when a sustained threshold titre of neutralising antibody is present in vivo (Parren et al., 2001). However, one draw-back to this approach is that a constant neutralising antibody titre must be maintained to provide this protection.

Work by Veazey et al. (2003) attempted to tackle the problem of needing constant high levels of circulating neutralising antibodies by designing a topical microbicidal containing the b12 MAb. A topical antibody-based microbicidal, could then be frequently used to protect against infection. Antibody (5mg) was administered into the vagina of female macaques and challenged 15 minutes later with 300TCID$_{50}$ of SHIV$_{162p4}$ (Veazey et al., 2003). Of the 12 animals challenged only 3 became infected, indicating that vaginal application of broadly neutralising antibody as a topical microbicidal can prevent infection (75% success rate) (Veazey et al., 2003).

1.9.2 HIV-1 gp160 antigenicity

The induction of broadly neutralising antibodies is essential to achieving sterilising immunity from vaccination, but to-date few broadly neutralising antibodies have been described (Ofek et al., 2004). It is difficult to elicit broadly neutralising antibodies in humans using recombinant soluble glycoproteins (Adis International Ltd, 2003; Francis et al., 2003), therefore, defining the epitopes which frequently produce broad neutralising antibody responses is crucial in the engineering of an HIV-1 immunogen able to provide protective immunity.

Antibodies against HIV-1 neutralise virus infectivity in one of two ways, by inhibiting virus-receptor interactions or by interfering with events post-receptor binding, for
example during membrane fusion (Ugolini et al., 1997; Huber et al., 2007). Most neutralising antibody activity is strain, making the development of a cross-clade HIV-1 vaccine difficult (Goudsmit et al., 1988; Burton et al., 2004). Ideally an HIV-1 vaccine should elicit multiple neutralising antibody species in order to combat emerging viral resistance, however, given the difficulty in producing such antibodies in the first instance, this is proving to be a challenge.

Some of the first broadly neutralising antibodies were produced by Roben et al. (1994), who isolated gp120 binding FAb fragments from a phage library produced from the DNA of a single HIV-1 infected donor. Six of the phage were characterised and the most potent neutralising MAb isolated was named b12 (Roben et al., 1994). The b12 MAb neutralises a range of primary and laboratory HIV-1 isolates and is specific for a discontinuous epitope within the CD4 binding site of gp120 (Moore et al., 1994; Moore et al., 1995). Figure 1.1 shows the antigenic structure of gp120 and the binding sites of the MAbs discussed.

This experimental method of isolating MAbs has proved invaluable for isolating other CD4 binding site antibodies that do not bind to the b12 epitope (Buchacher et al., 1994). In utilising the same library, whilst incubating phage with gp120 after preadsorption of highly potent CD4 binding site antibodies, like b12, a large number of alternative CD4 binding site targeting antibodies were isolated. This allowed the isolation of antibodies that bind to sites including the V2 region of gp120 (Buchacher et al., 1994).

The MAb 2G12 is unusual among antibodies as it binds to a conformation- and carbohydrate-dependent epitope of gp120 (Buchacher et al., 1994; Purtscher et al., 1994). Binding to the carbohydrate covered ‘silent face’ of the gp120 outer domain, 2G12 attaches to mannose residues on the distal end of conserved clusters of oligomannose sugars (Calarese et al., 2005). Despite its neutralising ability, the 2G12 antibody only partially neutralises HIV-1MN, a B clade isolate, and is unable to neutralise C clade HIV-1 isolates (Bures et al., 2002; Binley et al., 2004). Although the underlying protein surface influences the presentation of sugar residues important for 2G12 binding, there is no evidence of direct interaction between the antibody and these amino acid residues. However, binding of 2G12 is abolished by amino acid substitution
Figure 1.14. Antigenic map of HIV-1 gp160.

A. Space filled model of a gp120 monomer illustrating the different antigenic faces of gp120. The non-neutralising face is the site of interaction with other gp120 monomers, therefore, there is restricted access for antibody binding. The neutralising face represents an exposed region of gp120 which includes the V2 and V3 domains (B). This region interacts with the broadly neutralising MAbs targeted to the V3 loop, for example 447-52D. The silent face of gp120 is heavily glycosylated (B) and bind the MAb 2G12, which are directed to carbohydrate residues. C. this model of the gp120 monomer illustrates the common binding zones for 2G12, the CD4i MAbs like 4.8D and the CD4 binding site (CD4BS) which is the location of the b12 epitope. D. shows a representation of the MPER region of gp41. The 2F5 and 4E10 MAbs, directed against this MPER, neutralise HIV-1 by inhibiting viral fusion. Images A-C are adapted from (Wyatt et al., 1998), whilst D is adapted from (Song et al., 2009).
that results in the removal of the N-linked carbohydrate residues in the C2, C3, V4 and C4 regions of gp120 (Trkola et al., 1996). These areas, therefore, are implicated in creating the carbohydrate structure of the 2G12 epitope (Trkola et al., 1996; Sanders et al., 2002; Scanlan et al., 2002).

The MAbs 2F5 (Buchacher et al., 1994; Muster et al., 1994) and 4E10 (Muster et al., 1993; Buchacher et al., 1994) neutralise a wide range of HIV-1 isolates by preventing membrane fusion. They have been mapped to the exposed ectodomain within the membrane proximal exposed region (MPER) of gp41. This region is defined as the aromatic-rich, conserved gp41 sequence composed of gp41 amino acids 662-683 (Binley et al., 2004; Huarte et al., 2008).

The 2F5 MAb binds at a linear epitope (ELDKWAS) located near the C’terminal end of gp41 (Buchacher et al., 1994; Muster et al., 1994; Purtscher et al., 1994). The 4E10 core epitope spans gp41 amino acids 671 to 676 and binds to the consensus, NWF(D/N)IT (Zwick et al., 2001; Sun et al., 2008). Despite epitope linearity, antibody binding is conformation-dependent, as denaturation of gp41 reduces 4E10 binding (Zwick et al., 2001). Not all isolates possessing this sequence are neutralised by 4E10, indicating there are conformational and possibly other determinants within native Env that may influence epitope structure (Zwick et al., 2001). The 2F5 MAb neutralises a range of primary and laboratory-adapted HIV-1 isolates, with the exception of clade C viruses (Binley et al., 2004). Unlike 2F5, the 4E10 MAb can neutralise a broader range of HIV-1 isolates at an epitope within the gp41 MPER (Binley et al., 2004).

Interestingly, hydrophobic and conserved residues (W672, F673, L679) within the 4E10 epitope are immersed in the viral membrane and, therefore, antibody binding occurs by attaching to the exposed epitope residues before binding to the membrane immersed amino acids prior to viral neutralisation (Sun et al., 2008). Direct interactions also occur between the hydrophobic CDR3 region of 4E10 and the viral membrane (Alam et al., 2007). Lipid binding of 4E10 supports an induced fit, conformational model, described as initial lipid binding that induces binding to MPER epitopes, in turn leading to neutralisation (Alam et al., 2007). Both MPER neutralising antibodies, 4E10 and 2F5, have been shown to bind membrane lipids and form weak interactions with phospholipids, but stronger binding to cardiolipin (Alam et al., 2007). Non-neutralising
antibodies binding in the MPER region, such as 13H11, form no interactions with lipids illustrating the possible importance of membrane lipids in 4E10 and 2F5 neutralisation in particular (Alam et al., 2007).

Studies involving the presentation of MPER MAbs in proteoliposomes or lipid membranes have shown that lipids can alter the binding of these MAbs (Zwick et al., 2001; Grundner et al., 2002; Ofek et al., 2004; Haynes et al., 2005; Lorizate et al., 2006; Alam et al., 2007; Sun et al., 2008), suggesting that the antigenicity of HIV-1 glycoproteins is influenced by a lipid environment (Alam et al., 2007). Binding of broadly neutralising antibodies is sensitive to conformational changes induced upon anchoring gp41 peptides in synthetic lipid bilayers (Ofek et al., 2004; Alam et al., 2007). Therefore, the key to eliciting antibodies, like 2F5 and 4E10, may be to create lipid-associated Env immunogens.

The V3 domain of gp120 is highly exposed on the native gp160 trimer and readily elicits neutralising antibodies (Gorny et al., 1992). The Mab 447-52D is one such antibody, which neutralises a wide range of laboratory and field isolates containing the GPGR V3 motif (Conley et al., 1994). Despite the neutralising potential of 447-52D, the V3 region of gp120 is highly variable and, hence, viral escape from neutralisation by this antibody is common (Kwong, 2004).

The MAb 4.8D (Thali et al., 1993) is unusual in that it binds to an epitope that is exposed upon CD4 binding (thus named CD4-induced or CD4i antibodies). Due to the short time of the exposure of conserved sequences within gp120 after CD4 binding in vivo and the steric hindrance these antibodies must overcome to bind at these epitopes, they generally have poor neutralising ability (Labrijn et al., 2003). There are exceptions to this rule, however, as Labrijn et al. (2003) identified a unique CD4i, X5, which unlike others was found to neutralise a wide range of HIV-1 primary isolates. These CD4i antibodies bind at the conserved co-receptor binding site and are commonly produced in vivo (Decker et al., 2005).
1.9.3 T cell immunity

HIV-1 infects CD4+ T cells of the cellular adaptive immune system and within 2-3 weeks of infection some 50% of CD4+ CCR5+ T cells are infected and destroyed (Brenchley et al., 2004; Mehandru et al., 2004; Li et al., 2005; Mattapallil et al., 2005). This occurs predominantly in the gastrointestinal system of infected individuals (Brenchley et al., 2004; Mehandru et al., 2004; Li et al., 2005). Figure 1.2 illustrates the time course of HIV-1 infection with respect to CD4+ T cells. Virus-specific T_H cells can be detected in most patients with active or progressive HIV-1, but are at their most numerous during the latent, non-progressive chronic stage of HIV-1 infection where they are available to support anti-HIV-1 effector responses in active disease (Pitcher et al., 1999).

The CD4+ T cell plays an important role in controlling HIV-1 infection. Few CD4+ T cells in vivo actively produce the virus at any one given time, and those CD4+ T cells that are functional stimulate CD8+ cytolytic T cell responses through T-helper (T_H) cell functions (Cloyd et al., 2001). The CD4+ CCR5+ T cells, in particular, are important for CD8+ T cells (cytotoxic lymphocytes, CTLs), producing the cytokines (RANTES, MIP-1α and MIP-1β) required for the stimulation of an effective cellular immune response to infection (Hadida et al., 1998; Lehner et al., 2000). Eventually, viraemia overcomes immune regulation causing CD4+ T cell depletion through productive infection or indirectly killing cells via the ‘bystander effect’ (apoptosis of non-infected cells) (Cloyd et al., 2001). Therefore, regardless of the positive role CD4+ T_H cells play in anti-HIV responses, other factors contribute to the eventual decline in CD4+ T cells, coinciding with the onset of AIDS-related symptoms.

Individuals who control viraemia in the absence of HAART, such as LTNPs, have been shown to have persistant polyclonal CD4+ T_H cell proliferative responses to HIV-1 proteins (Sheppard et al., 1993; Cao et al., 1995; Pantaleo et al., 1995; Rosenberg et al., 1997; Rosenberg et al., 2000). Proliferative responses of CD4+ T_H cells to p24 during chronic infection are inversely proportional to viral load, indicating the importance of this cellular component of the immune response during HIV-1 infection (Rosenberg et al., 1997; Rosenberg et al., 2000). Both helper and effector CD4+ T cells are
consistently found in LTNPs, which indicates their importance in combating viral infection (Sheppard et al., 1993; Cao et al., 1995; Pantaleo et al., 1995).

Activation of CD8$^+$ T cells relies on a viable CD4$^+$ T cell population, which stimulate the generation and maintenance of antigen-specific CTL cells during chronic infection (Rosenberg et al., 1997; Rosenberg et al., 2000). Treatment of HIV-1 by HAART during acute infection can augment CD4$^+$ T cell responses and, in turn, can increase the CTL response in patients (Rosenberg et al., 1997; Rosenberg et al., 2000). This allows the activation and maintenance of CTL immunity (Rosenberg et al., 1997; Rosenberg et al., 2000). Control of viraemia during early infection rests with the CD8$^+$ cytotoxic T lymphocyte. HIV-specific CD8$^+$ T cells develop quickly and are responsible for the destruction of HIV-infected cells (Borrow et al., 1994). This is shown by the experimental depletion of CTLs in primary infection in macaques, which results in uncontrolled virus replication (Schmitz et al., 1999). The CTL is also important during chronic infection. Elimination of these cells in chronic infection in an SIV macaque model led to a rapid increase in viraemia (Schmitz et al., 1999), which was subsequently suppressed by the reappearance of SIV-specific CTL cells, thus confirming their importance in viraemia control (Schmitz et al., 1999).

Patients that mount a strong gp160-specific CTL response exhibit rapid reduction of acute plasma viraemia compared to those which poorly controlled viraemia with low numbers of specific CTLs. Therefore, the CTL component of the immune system is responsible for the control of virus replication following primary infection (Borrow et al., 1994). However, it has not been possible to demonstrate a direct relationship between CTL activity and plasma RNA viral load, unlike the relationship described between viral load and the number of CD4+ T cells (Rosenberg et al., 1997; Ogg et al., 1998; Rosenberg et al., 2000).

A panel of HIV-1 positive individuals (n=57) has been screened for cellular responses to Tat and Rev using overlapping peptides (Addo et al., 2001). The percentage of patients exhibiting cellular response to Tat and Rev were 19% and 37%, respectively, with multiple CTL epitopes in each protein (Addo et al., 2001). Cellular responses to RT have also been found in the blood of HIV-1 infected individuals, indicating that
immunity to proteins other than Env and Gag are also important during infection (Walker et al., 1987; Walker et al., 1988; Addo et al., 2001).

Despite the wide ranging CTL response to infection, amino acid mutations frequently accumulate and can select for new viral escape variants during acute infection (Allen et al., 2000; Addo et al., 2001). As viral mutation is common place, this can undermine CD8+ T cell control of HIV-1 infection (Roberts et al., 1988; Mansky et al., 1995).

Although an array of cells from the adaptive and innate immune system contribute to controlling HIV-1 infection, only two of the cells involved in the cellular adaptive immune response have been discussed in this section, those involved in cell-mediated immune responses thought to be key players in potential HIV-1 vaccines.

1.10 HIV-1 inactivated vaccine strategies

Since the majority of vaccines approved for use worldwide are based on inactivated viruses, bacteria or toxins, it is important to revisit the idea of a whole-inactivated HIV-1 immunogen, despite concerns over the safety of such an approach. The development of an inactivated HIV-1 vaccine could answer questions about the nature of a protective immune response against HIV-1 and how to stimulate it.

1.10.1 Inactivating agents

There are many chemical agents that can successfully reduce HIV-1 infectivity in vitro (Murphrey-Corb et al., 1989; Race et al., 1995; Race et al., 1995; Rice et al., 1995; Rice et al., 1997; Arthur et al., 1998; Grovit-Ferbas et al., 2000; Miller Jenkins et al., 2005). Some of these chemical inactivating agents are explored in Chapter 5 in the production of cholesterol-depleted, whole-inactivated HIV-1MN with preserved gp160 conformation. With the production of an HIV-1 immunogen by inactivation, two key factors are important for vaccine studies. Firstly, the agent must be capable of destroying viral infectivity and, secondly, the inactivation process must retain conformational epitopes important in eliciting antibodies to HIV-1.
Methods of inactivating HIV-1 utilising agents that are activated by light have also been explored *in vitro* and *in vivo* as anti-retroviral agents. For example, irradiation (UV, X-ray and photo-activated inactivation compounds) has been utilised as a method of reducing HIV-1 infectivity (Henderson *et al.*, 1992; Vyas *et al.*, 2002; Raviv *et al.*, 2005). Photo-inactivation using the compound, 1, 5-iodonaphthylazide (INA), which is activated by UV light, by alkylation of integral membrane proteins, such as gp41 (Raviv *et al.*, 2005). Dimethyl methylene blue (DMMB) is another compound that can ablate HIV-1 infectivity upon exposure to fluorescent light (Vyas *et al.*, 2002). Both of these compounds, activated by ultra-violet or X-ray radiation, can be used to reduce HIV-1 replication by causing genomic mutation (Henderson *et al.*, 1992).

There have been interesting developments in the use of inactivating agents in therapeutic strategies. Yamaguchi *et al.* (2008) have developed titanium oxide (TiO$_2$) particles, which on irradiation with UV light produce electron holes catalysing redox reactions with organic matter. Conjugation of CD4 to these particles has allowed the selective inactivation of HIV-1 (by over 90%). These particles have been designed as a therapeutic immunophysical strategy in which, CD4-TiO$_2$ is administered to macaques and blood plasma from live animals exposed to UV radiation (by extra-corporeal circulation) (Yamaguchi *et al.*, 2008). Animals with viral loads of 500 TCID$_{50}$/ml underwent a reduction in circulating virus to zero upon exposure of blood to UV light before reinfusion. Four to seven weeks after treatment, the viral load set point was reduced compared to untreated controls. The advantages of this system include the fact that TiO$_2$ is not toxic and only activated by UV light. As this method of inactivation non-specifically targets all viral proteins, it may have important applications when drug resistance prevents conventional HAART therapy.

Hydrostatic pressure (300MPa) has also be used to inactivate HIV-1 infectivity by up to 6 logs (Meyer *et al.*, 2002). Vaccination of macaques, with HIV-1$_{F-SI}$ inactivated by hydrostatic pressure, proved to elicit cytokines and antibodies (Meyer *et al.*, 2002). Hydrostatic pressure has been used for creating vaccines against adjuvant arthritis autoimmunity and cancer (Lider *et al.*, 1987; Goldman *et al.*, 2000).
1.10.2 Whole-inactivated retroviral vaccines

The use of inactivated retroviruses as immunogens in animal models has involved lentiviruses common to species other than humans, such as Simian Retroviruses (SRVs), Equine Infectious Anaemia virus (EIAV) and Feline Immunodeficiency virus (FIV) and given insight into HIV-1 immunogens. These viruses mimic HIV-1 disease presentation, progression, the immune responses to infection and antigenic variation (Dunham, 2006; Dunham et al., 2006).

Simian retrovirus (SRV) is a type D simple retrovirus discovered in 1984 in rhesus macaques (Marx et al., 1984). This virus causes simian acquired immunodeficiency syndrome (SAIDS) in macaques, which mimics closely the infection of humans with HIV-1 (Marx et al., 1984). Vaccination of monkeys with formalin-inactivated SRV-1 vaccination protected them from challenge with lethal doses of SRV-1. All vaccinated animals developed neutralising responses, lending support to the idea that protection against infection with lentiviruses can be provided by whole-inactivated vaccines (Marx et al., 1986). These experiments were an early example of the potential of inactivated retroviral vaccines.

In a similar way to SRV, the use of retroviruses like equine infectious anaemia virus (EIAV) and feline immunodeficiency virus (FIV) have also demonstrated potential in inactivated vaccine approaches. A formalin-inactivated EIAV vaccine in ponies has provided protection against challenge with high doses ($10^6$ TCID$_{50}$) of homologous viral strains with the development of both humoral and cell-mediated immunity, including neutralising antibodies (Issel et al., 1992). Despite the failure to protect against heterologous EIAV strains, formalin-inactivated EIAV prevented clinical disease progression in these animals, highlighting the possible applications of therapeutic vaccine strategies in the control of disease progression (Issel et al., 1992).

Vaccination using inactivated FIV can provide protection against homologous virus challenge (Hosie et al., 1995). In this study by Hosie et al. (1995), partial protection was also provided against less virulent heterologous strains. This corresponded to a reduction in viral load along with the maintenance of CD4$^+$ and CD8$^+$ T cells on infection after virulent heterologous strain challenge (Hosie et al., 2000). These data
suggest that, given the antigenic diversity among HIV-1 isolates, cross-clade protection with a single clade vaccine may be hard to achieve, but may be possible with careful tailoring to clade requirements.

Early work in the 1980’s, involving formalin whole-inactivated SIV/DELTA<sub>B670</sub> preparations was promising, as the majority of animals (8 of 9) challenged post-vaccination were protected from infection (Murphey-Corb <i>et al.</i>, 1989). As antibody production was lower than that seen during infection, protection did not correlate with humoral responses and, therefore, the true nature of protective immunity post-vaccination was unclear (Murphey-Corb <i>et al.</i>, 1989). It was later identified that immune responses to cellular proteins resulting from propagation of virus in human cell lines (xenoantigens) were the cause of this protection (Stott, 1991). Upon challenge with SIV<sub>mac</sub> cultivated in rhesus PBMC’s, protection was lost (de Vries <i>et al.</i>, 1994).

Other studies involving whole-inactivated retroviral immunogens have yielded mixed results. Desrosiers <i>et al</i> (1989) showed that formalin-inactivated and disrupted SIV resulted in little protection against infection in macaques (2 of 6 animals protected), despite observations of specific humoral immunity including neutralising antibodies. Despite the lack of protection, those animals which became infected after challenge did demonstrate a decreased viral burden (Desrosiers <i>et al.</i>, 1989).

Vaccination of macaques with psoralen-UV-light inactivated whole SIV<sub>mac</sub> did not protect animals from infection, but led to delayed clinical course (Sutjipto <i>et al.</i>, 1990). A study by Stahl-Hennig et al. (1992) only demonstrated protection in 57% of animals after immunisation with Tween-disrupted SIV<sub>mac251</sub> adsorbed to Alum adjuvant. This preparation protected 4 of 7 animals vaccinated resulting from antibody and cell-mediated responses in those protected (Stahl-Hennig <i>et al.</i>, 1992). In the Stahl-Hennig study, however, the challenge dose used was one third to one quarter of that used after formalin-inactivated and psoralen-UV-light inactivated SIV<sub>mac</sub> immunisation by Sutjipto <i>et al.</i> (Stahl-Hennig <i>et al.</i>, 1992). This illustrates that the differences in protection success in different animal studies is highly subjective depending on the challenge dose and highlights the need to standardise SIV immunogen testing in animal models. Moreover, using a range of challenge doses could indicate both the ability of a vaccine to protect against infection and the robustness of the immune response.
Recent investigation into whole-inactivated vaccines has utilised SIV\textsubscript{mac}E11S strains that possess greater amounts of surface glycoprotein, and mutant SIV\textsubscript{mac239} which has deleted carbohydrate attachment residues, both chemically inactivated by aldrithriol-2 (AT-2). The inactivating agent AT-2 oxidises amino acids in the zinc finger domains of the NC protein, ejecting the chelated Zn\textsuperscript{+} ions (Miller Jenkins \textit{et al.}, 2005). This ablates HIV-1 infectivity before reverse transcription as NC plays a vital role in this process (Miller Jenkins \textit{et al.}, 2005). Protection against challenge was provided for 4 of 6 animals with homologous SIV\textsubscript{mac}, but not heterologous SIV\textsubscript{smE660}, and resulted from both antibody and cell-mediated immune responses (Lifson \textit{et al.}, 2002; Lifson \textit{et al.}, 2004). These results point to the possibility that whole-inactivated HIV-1 vaccines may be effective, given inactivation with agents that maintain protein antigenicity and maximise the gp160 immunogen content.

Removing cholesterol from HIV-1 viral envelopes is useful in altering the antigenicity and immunogenicity of whole-inactivated immunogens. Cholesterol depletion affects whole-inactivated SIV vaccination \textit{in vivo}, indicating the importance of protein presentation in the context of the viral envelope (Kitabwalla \textit{et al.}, 2005; Kitabwalla \textit{et al.}, 2008). Lipid (cholesterol, triglyceride and unesterified fatty acid) depletion of SIV\textsubscript{MAC-251} using the solvent treatment, di-isopropyl ether (DIPE), has been investigated for the purpose of creating a lipid-depleted, inactivated SIV\textsubscript{MAC-251} vaccine (Kitabwalla \textit{et al.}, 2005). Solvent treatment resulted in 80\% removal of the viral envelope cholesterol and reduced infectivity by 2 logs, but had minimal effect on viral protein composition (Kitabwalla \textit{et al.}, 2005). Observations of the maintenance of protein composition in SIV\textsubscript{MAC-251}, while reducing viral envelope lipid content and, hence, viral infectivity are similar to those found in the treatment of HIV-1 with β-cyclodextrins (Liao \textit{et al.}, 2001; Graham \textit{et al.}, 2003).

Murine studies which investigated the cell-mediated and humoral immunity to delipidated inactivated SIV\textsubscript{MAC-251} elicited greater humoral and cell-mediated immune responses to vaccination compared to inactivated SIV\textsubscript{MAC-251} alone (Kitabwalla \textit{et al.}, 2005; Kitabwalla \textit{et al.}, 2008). Initial prime immunisation with inactivated SIV\textsubscript{MAC-251}, followed by a further immunisation using delipidated SIV\textsubscript{MAC-251} (boost), produced elevated antibody titres (Kitabwalla \textit{et al.}, 2005). However, the vaccination schedule
was designed primarily to investigate cell-mediated responses and, therefore, the short time between boost and sample collection (4 days after boost) was sub-optimal for antibody titre evaluation (Kitabwalla et al., 2005).

Cell-mediated immunity, characterised by an increased breadth of SIV<sub>MAC-251</sub> peptide recognition of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells, increased as a result of vaccination with delipidated SIV<sub>MAC-251</sub> (Kitabwalla et al., 2005). This effect was Env-specific, as Gag peptide responses for delipidated-inactivated SIV<sub>MAC-251</sub> and inactivated SIV<sub>MAC-251</sub> alone were similar. However, mice primed with saline and boosted with delipidated SIV<sub>MAC-251</sub>, showed no detectable T cell responses, suggesting a single inoculation was insufficient to elicit cell-mediated immunity (Kitabwalla et al., 2005).

In a proof-of-concept pilot experiment in macaques, virus was isolated from SIV<sub>MAC-239</sub> chronically-infected animals and used to create an autologous, lipid-depleted vaccine (Kitabwalla et al., 2008). Macaques were treated with anti-retroviral PPMP ((R)-9-[2-(phosphonomethoxy)propyl]adenine) chemotherapy during the vaccination schedule and cell-mediated immune responses, on cessation of anti-retroviral treatment, monitored (Kitabwalla et al., 2008). Vaccinated groups (receiving 3 innoculations, one month apart) showed increased survival compared to unvaccinated controls infected with identical SIV strains (Kitabwalla et al., 2008). This corresponded to an increase in the breadth of CD8<sup>+</sup> T cell responses to SIV Env and Gag peptides. Although greater breadth of CD4<sup>+</sup> T cell responses were also observed, this result was not significant (Kitabwalla et al., 2008). These data show that a change in viral envelope lipid composition may influence vaccine responses.

### 1.10.3 Inactivated HIV-1 vaccines

Investigation of HIV-1 whole-inactivated vaccines is an area that has not been thoroughly explored. Some in vivo studies have shown the possibility of raising both neutralising antibody and cell-mediated immune responses to inactivated HIV-1 and are discussed below. Race et al (1995) used a combination of inactivating agents to validate the safety and immunogenicity of the whole-inactivated virus approach with
respect to HIV-1. Laboratory-adapted strains of HIV-1 were extensively inactivated with a combination of inactivating agents including BPL or with BPL alone and administered in murine studies (Race et al., 1995; Race et al., 1995). Promising homologous and heterologous neutralising antibody responses (that were not due to cellular antigens) were detected, which suggested that important viral epitopes were preserved during the inactivation process (Race et al., 1995; Race et al., 1995).

In a separate study, vaccination of mice with HIV-1SX, inactivated using 0.02% formalin and 62°C heat treatment, elicited neutralising antibody responses to homologous or similar B-clade laboratory-adapted strains (HIV-1SF and HIV-1NL4-3) (Poon et al., 2005). Immunisation of non-human primates with the same inactivated HIV-1SX also produced neutralising antibody responses to B-clade viruses, but only after the third vaccination, illustrating the difficulty of producing neutralising antibody responses in vivo (Poon et al., 2005).

This same group published immunogenicity data obtained from mouse and rabbit studies using a formalin and heat-inactivated HIV-1SX (O'Brien et al., 1990) mutant strain, possessing ten times the average amount of surface gp120 (Poon et al., 2005). Whole viral immunogens retained neutralising epitopes upon inactivation and assessment of antibody binding showed enhanced antigenicity compared to wild type HIV-1SX (Poon et al., 2005). Mice were vaccinated with this inactivated HIV-1SX mutant strain and the sera collected upon sacrifice (after 4 vaccinations) tested against a panel of HIV-1 isolates of diverse clades (A, B, C and A/E clades) for neutralising antibody responses (Poon et al., 2005). Antibody binding titres of greater than 1/1000 were observed against cross-clade HIV-1 isolates, as well as neutralising antibody titres of 1 in 100 against the clade B viruses (Poon et al., 2005). However, after immunising rabbits with 6 doses of inactivated mutant HIV-1SX, neutralising antibody titres only ranged from 1 in 20 to 1 in 640 for the panel of HIV-1 isolates tested (Poon et al., 2005).

A formalin-inactivated HIV-1 vaccine, inoculated into chimpanzees, has been shown to elicit specific antibody responses in all animals, but with little neutralising ability or variation among cell-mediated responses (Niedrig et al., 1993). Upon challenge, all
animals became infected upon challenge, however, a high challenge dose was used (Niedrig et al., 1993).

These experimental studies show that, firstly, antibody immune responses to whole-inactivated HIV-1 immunogens can be elicited in mouse, rabbit and primate animal models. Secondly, that antibodies resulting from immunisation with inactivated HIV-1 can be broadly active against different clades and, thirdly, that neutralising antibodies can be stimulated by inactivated HIV-1 immunogens, but to a lesser extent that non-neutralising antibodies.

1.10.4 Other HIV-1 vaccine strategies

Many vaccine strategies have been used to produce potential HIV-1 immunogens. An overview of the different strategies used in the investigation of potential HIV-1 vaccines is discussed here, with examples of studies to illustrate the work being carried out.

**Recombinant protein or protein subunits**

The simplest vaccine to make would be the recombinant protein, or protein subunit vaccine, but vaccination using HIV-1 recombinant glycoproteins has proved unsuccessful. Although many studies have demonstrated cellular and humoral immunity to vaccination with recombinant monomeric gp120 or gp120 extracted from HIV-1 infected cell membranes, these attempts have failed to protect against virus challenge and exhibited little effect on subsequent viral load set points post-challenge (Berman et al., 1988; Arthur et al., 1989; Kumar et al., 2000).

Some studies have proved to be more successful. For example, vaccination of rhesus macaques with SIV Env peptides that are hydrophilic, immunoreactive and homologous with conserved regions of HIV-1 Env has produced peptide-specific neutralising antibodies post-vaccination (Shafferman et al., 1991). Although animals were not protected against infection, CD4+ T cells counts in vaccinated macaques did remain stable for the duration of the experiment (80 weeks) (Shafferman et al., 1991). The lack of protection by recombinant protein or protein subunit vaccines results from the
need for native gp160 glycoprotein trimers, perhaps even in the context of the viral envelope in order to elicit a relevant immune response that could protect against infection (Luke et al., 1996; Barnett et al., 2001; Cho et al., 2001).

Protein vaccination of macaques using a combination of HIV-1 proteins including gp120, Nef-Tat fusion proteins and SIV Nef has also been explored with little success in preventing infection on challenge (Voss et al., 2003). Despite this, partial protection was observed as a reduction in viral load, protection against CD4+ T cell depletion and prevention of AIDS for more than 2 and a half years did indicate the promotion of immune responses protective against disease progression (Voss et al., 2003). This work pointed to the use of HIV-1 glycoproteins in combinations with other viral proteins to give a more effective immune response against disease progression, but not sterilising immunity.

**Attenuated HIV-1**

The most attractive vaccine strategy is that of the attenuated vaccine. Vaccines that take this approach, for example the BCG tuberculosis vaccine, the Sabin attenuated poliovirus vaccine or cowpox variolation to prevent smallpox, have been highly successful. These immunogens retain the ability to replicate, all be it in a limited fashion, and can produce robust protective immune responses *in vivo* (Baba et al., 1995).

A Nef-deleted, attenuated SIV immunogen has been thoroughly explored in animal models (Marthas et al., 1990; Daniel et al., 1992; Norley et al., 1996; Hofmann-Lehmann et al., 2003). Macaques previously infected with SIV\textsubscript{mac} ΔNef were shown to be fully protected against wild type virus superinfection. However, this protection was not developed until 20 weeks post-infection, indicating a fully protective immune response is slow to develop (Daniel et al., 1992). Despite the success of these experiments in demonstrating the effectiveness of attenuated SIV in protecting against superinfection and AIDS-related disease, Baba et al. (1995) demonstrated that in neonatal macaques attenuated Nef-deleted SIV could grow to high titre and cause AIDS (Baba et al., 1995).
Other attenuated SIV isolates include an infectious, virulence attenuated SIV clone (SIV\textsubscript{mac-1A11}). This attenuated SIV strain caused 1 to 6 weeks of transient viraemia in infected macaques without presentation of clinical disease (Marthas \textit{et al.}, 1990). Animals infected with SIV\textsubscript{mac-1A11} had a persistent antibody response and upon challenge with pathogenic SIV\textsubscript{mac} became viraemic (Marthas \textit{et al.}, 1990). Despite this, animals displayed no sign of illness for 304 days compared to 260 days in unimmunised macaques (Marthas \textit{et al.}, 1990).

Similarly, Hofmann \textit{et al.} (2003) produced an attenuated SIV strain with major deletions in Nef, Vpr and genomic negative regulatory elements. This attenuated SIV strain was able to infect macaques and in time cause immune dysregulation in most animals. However, immune dysfunction did not occur until several years after vaccination (Hofmann-Lehmann \textit{et al.}, 2003). Therefore, it is apparent that attenuated strains of SIV tend to confer complete immune protection and can cause infection, albeit without manifestation of disease.

**Virus-like particles**

The importance of maintaining a native viral particle has led to the exploration of virus-like particles (VLPs) as HIV-1 immunogens. Chimeric VLPs containing proteins of HPV (L1), SIV (p27) and HIV-1 (Tat and Rev) have been used to immunise macaques systemically and mucosally (Dale \textit{et al.}, 2002). These VLPs were combined with a priming DNA vaccine expressing identical antigens. Only a weak antibody response and T-cell responses were elicited post-immunisation, providing no protection against viral challenge (Dale \textit{et al.}, 2002).

Vaccination of SIV-infected rhesus monkeys with pseudotyped non-infectious HIV-1 VLPs with the incorporation of VSV-G glycoprotein produced a 100-fold increase in antibody titre to HIV-1 Gag and enhanced T cell responses, compared to VLPs without a glycoprotein element (Kuate \textit{et al.}, 2006). Immunised animals were challenged mucosally post-vaccination and despite an initial decrease in peak viraemia, persistent suppression of viral load was not achieved (Kuate \textit{et al.}, 2006).
DNA strategies

More recently, attention has focused on DNA vaccine technology. Infection of rhesus macaques with proviral DNA has been shown to provoke similar immune response patterns compared to natural infection, however, immune responses to proviral DNA infection were delayed (Busch et al., 2003). These data show that DNA can provoke immune responses in vivo. Moving towards a possible vaccine, Singh et al. (2005) produced a plasmid DNA vaccine containing a full SIV/HIV immunodeficiency virus (SHIV), SHIV\textsubscript{KU2}, genome rendered non-infectious by the deletion of the RT gene. Rhesus macaques were immunised at week 0, 8 and 18, with all animals demonstrating antibody and cell-mediated immune responses post-vaccination (Singh et al., 2005). Vaccinated animals were challenged with heterologous SHIV and all became infected, however, lower viral loads were maintained in vaccinated individuals compared to control unvaccinated animals (Singh et al., 2005).

Similarly, a non-infectious full genomic DNA vaccine, with mutations in NC, elicited specific immune responses in macaques as a result of vaccination (Akahata et al., 2003). Although all animals became infected on challenge, 2 to 3-fold reductions in viral load were observed in vaccinated animals compared to the controls (Akahata et al., 2003). Moreover, viral load fell to below detectable limits in vaccinated animals 6 weeks post-challenge, indicating partial protection against disease progression (Akahata et al., 2003).

Many studies have been carried out on DNA vaccination that utilise plasmids containing combinations of SIV and/or HIV-1 proteins (Barouch et al., 2000; Kim et al., 2001; Muthumani et al., 2003). Although no DNA immunisation study has produced sterilising immunity upon challenge, induction of immune responses in vaccinated animals is frequently observed (Barouch et al., 2000; Kim et al., 2001; Muthumani et al., 2003). It is clear that these immune responses cannot prevent infection, but they may enable increased immune control of viral replication, along with preservation of CD4\textsuperscript{+} T cell populations and a delay in progression of disease post-immunisation (Barouch et al., 2000; Kim et al., 2001; Muthumani et al., 2003).
**HIV-1 protein expressing vectors**

As the development of an attenuated HIV-1 virus has been unsuccessful and with the substantial risk of reversion to wild type, the vector vaccine strategy has been widely explored. Numerous different viruses have been exploited for this purpose, including poxviruses, adenoviruses and the Herpes Simplex virus (HSV) (Buge et al., 1999; Kent et al., 2000; Murphy et al., 2000; Earl et al., 2002; Santra et al., 2002; Suh et al., 2006; Kaur et al., 2007).

Both replication competent and replication defective HSV expressing SIV viral proteins including Env, Nef, Tat and Rev have been tested in a macaque model (Murphy et al., 2000; Kaur et al., 2007). Although both humoral and cellular immune responses have been demonstrated, full protection from SIV infection was not achieved (Murphy et al., 2000; Kaur et al., 2007). However, as seen with DNA vaccination vaccinated animals were better able to control viraemia levels compared to controls (Murphy et al., 2000; Kaur et al., 2007).

Another popular group of viruses used in development of HIV-1 vector vaccines is the poxviruses. Fowlpox and avipox vectors containing HIV-1 and SIV proteins, respectively, have been used to determine if vector vaccination could help control viral replication as a therapeutic vaccine (Kent et al., 2000; Santra et al., 2002). These vectors have been shown to be safe and to produce immune responses in macaques following vaccination (Kent et al., 2000; Santra et al., 2002).

**Prime-boost strategies**

Although vectors expressing HIV-1 and SIV proteins can induce immune responses *in vivo*, prime-boost strategies involving vector vaccines combined with recombinant protein or DNA immunogens have emerged as being more effective than single vaccine approaches (Buge et al., 1999; Earl et al., 2002; Horton et al., 2002; Patterson et al., 2003; Radaelli et al., 2003; Suh et al., 2006). For example, priming of macaques with recombinant vaccinia virus expressing full length gp160 combined with a glycoprotein subunit boost was more effective in eliciting anti-viral immune responses than the subunit vaccine alone (Polacino et al., 1999).
Despite the superiority of prime-boost vaccine approaches not one potential HIV-1 immunogen has been able to prevent infection upon challenge. Although this is disappointing in terms of protective vaccine development, demonstration of a delay disease progression to AIDS and reduced viral loads in infected animals post-challenge holds promise for therapeutic vaccination (Buge et al., 1999; Earl et al., 2002; Horton et al., 2002; Suh et al., 2006). With adaptation of immunogens to optimise the immune response after immunisation, a potential vaccine for HIV-1 is a real prospect.

1.11 HIV-1 vaccine clinical trials

Numerous Phase I-III HIV-1 vaccine trials in humans have been undertaken to date and all have failed to provide a 100% protective immune response against infection. Table 1.2 details the current HIV-1 vaccine phase II and III clinical trials. Two Vaxgen Phase III clinical trials (VAXGEN 003 in North America and The Netherlands and 004 in Thailand) completed in 2003 investigated a recombinant monomeric gp120 immunogen in healthy individuals to assess immune responses elicited post-vaccination. No protective effect as a result of vaccination was observed in any of the participants (Gilbert et al., 2005; Iaccino et al., 2008). The study did seem to reduce the incidence of infection in certain ethnic populations, however, the validity of subset analysis within this trial has been questioned (Gilbert et al., 2005; Iaccino et al., 2008).

In 2004, a phase III clinical trial tested a gp120-depleted whole-inactivated vaccine (“REMUNE”) for therapeutic use in infected individuals (Chantratita et al., 2004). Despite some increases in CD4+ T cell function, this study also failed to demonstrate any increase in HIV-1 progression-free survival (Chantratita et al., 2004). This is not surprising given the key HIV-1 immunogen, gp160, was depleted from this potential vaccine, but did show for the first time that antibodies to gp160 were important.

A major setback in large scale phase III clinical trials of HIV-1 vaccine immunogens came when the Merck Trial of a recombinant adenovirus serotype 5 expressing gag, pol and nef genes was stopped prematurely. This trial was halted after the emergence at interim analysis that HIV-1 infection rates had increased in the vaccinated group (Iaccino et al., 2008).
Table 1.2. Current phase II/III HIV-1 vaccine trials

<table>
<thead>
<tr>
<th>Phase</th>
<th>Trial title</th>
<th>Vaccine strategy; antigen (clade)</th>
<th>Start Date</th>
<th>Countries (# of sites)</th>
</tr>
</thead>
<tbody>
<tr>
<td>III</td>
<td>A trial of Sanofi Pasteur live recombinant ALVAC-HIV (vCP1521) priming with Vaxgen gp120 B/E (VaxGen B/E) boosting.</td>
<td>Live recombinant prime; <em>env</em> (B,E) Protein boost; <em>gag/pol, env</em> (B, E)</td>
<td>Oct ’03 Finished ’09</td>
<td>Thailand (8)</td>
</tr>
<tr>
<td>II</td>
<td>A clinical trial to evaluate the safety and immunogenicity of a multi-clade HIV-1 DNA plasmid vaccine, VRC-HIVDNA-016-00-VP, followed by a multiclade recombinant adenoviral vector HIV-1 vaccine boost, VRC-ADV-014-00-VP.</td>
<td>DNA prime; <em>gag, pol, nef</em> (B), <em>env</em> (A,B,C). Vector boost; <em>gag, pol</em> (B), <em>env</em> (A,B,C).</td>
<td>Sept ’05</td>
<td>USA (7), Brazil (2), South Africa (3), Later, Haiti, Jamaica</td>
</tr>
<tr>
<td>I/II</td>
<td>A trial to compare the immunogenicity and safety of 3 DNA C primes followed by 1 NYVAC C boost to 2 DNA C primes followed by 2 NYVAC C boosts.</td>
<td>DNA prime; <em>env, gag, pol, nef</em> (C) Vector boost; <em>env, gag, pol, nef</em> (C)</td>
<td>Aug ’07</td>
<td>France (5), UK, Switzerland, Germany</td>
</tr>
<tr>
<td>I/II</td>
<td>A randomised, placebo-controlled, double-blind, clinical trial to evaluate the safety and immunogenicity of a candidate prophylactic, pHIS-HIV-AE prime followed by rFPV-HIV-AE boost (a non-replicating, recombinant fowlpox virus vector delivering the same genes as prime).</td>
<td>DNA prime; <em>gag, pol, tat/rev, env</em> (A, E) Vector boost; <em>gag, pol, tat/rev, env</em> (A, E)</td>
<td>Feb ’07</td>
<td>Thailand</td>
</tr>
<tr>
<td>I/II</td>
<td>A clinical trial to assess the safety and immunogenicity of a plasmid DNA-MVA prime-boost HIV-1 vaccine candidate</td>
<td>DNA prime; <em>env</em> (A, B, C), <em>gag</em> (A, B), RT (B), <em>rev</em> (B) Vector boost; <em>env</em> (E), <em>gag</em> (A), <em>pol</em> (E)</td>
<td>Dec ’06</td>
<td>Tanzania</td>
</tr>
</tbody>
</table>

Table 1.2. describes the current phase II and III HIV-1 vaccine clinical trials. Details of the trial title are shown along with the vaccine strategy, antigen and clade used. The trial start date and the country in which the trial is being carried out are also shown. The phase III trial highlighted in red has just been completed with modest efficacy results which are discussed on page 90.
Despite the failure of these phase III clinical trials, key lessons have been learnt. The most important of these is that although these immunogens were initially tested in animal models, these experiments were dismissed, as macaques models of SIV and SHIV were thought to be poor mimics of what may be achieved in humans (Barouch et al., 2000; Shiver et al., 2002). Therefore, the general consensus was that only human trials could indicate HIV-1 vaccine efficacy (Iaccino et al., 2008). However, in retrospect these preliminary animal experiments could have led to questions regarding the outcome of these trials.

The first phase III trial to report modest efficacy in preventing HIV-1 infection has just been reported by the US Military HIV-1 research program (MHRP) (Table 1.2). A trial of Sanofi Pasteur live recombinant ALVAC-HIV (vCP1521) expressing HIV-1 env from clades B and E followed by immunisation with Vaxgen gp120 B/E (VaxGen B/E) over 6 months has shown a 31.2% reduction in the number of participants infected with HIV-1 after 3 years of follow up, compared to the placebo control arm (Rerks-Ngarm et al., 2009). These results are extremely promising and could be important in the future direction of HIV-1 vaccine research.

1.12 Aims

No effective vaccine against HIV-1 currently exists. Therefore, it is important to explore all avenues in order to create such a vaccine, including traditional methods currently used for vaccine production, such as an inactivated vaccine. In the 1970’s, experiments showed that manipulation of the cell membrane lipid and cholesterol concentration could increase the antigenicity and immunogenicity of a whole cell cancer vaccine (Shinitzky et al., 1979). This was thought to result from changes in protein presentation at the cell surface (Borochov et al., 1976). Therefore, the purpose of this thesis is to determine the impact of viral envelope cholesterol content on the antigenicity of HIV-1 with the aim of creating a whole-inactivated HIV-1 immunogen with enhanced antigenicity and immunogenicity.

The aims of this thesis are to:
1. Assess the impact of cholesterol depletion on HIV-1MN infectivity, morphology, protein content, antibody binding and viral neutralisation.
2. Screen a panel of primary HIV-1 isolates with a view to selecting an isolate with high levels of gp120 for investigating the possibility of using primary isolates as potential whole-inactivated immunogens.

3. Assess the amount of virus-associated gp120 at different time points during infection.

4. Utilise a pseudotyped primary HIV-1 isolate to explore the effect of cholesterol depletion on primary isolate infectivity, protein content and viral neutralisation.

5. Determine the best inactivation method for producing an cholesterol-depleted, whole-inactivated HIV-1\textsubscript{MN} immunogen with conserved glycoprotein conformation.

6. Study the effect of cholesterol depletion on immune responses to whole-inactivated HIV-1\textsubscript{MN} in a mouse model.
Chapter 2:

Materials and Methods
2.1 Cell lines

Human cutaneous T-cell lymphoma (H9) cells (Popovic et al., 1984; Mann et al., 1989) were cultured using serum-free AIMV medium (Invitrogen).

Human T-lymphoblastoid C8166 cells (Salahuddin et al., 1983) were cultured in Roswell Park memorial Institute (RPMI) medium supplemented with L-glutamine (GIBCO), 10% (v/v) fetal calf serum (FCS) and 1% (v/v) penicillin/streptomycin (complete RPMI).

The NP2 cell line (Soda et al., 1999) is an adherent kidney cell line and is stably transfected with either the CD4 and CXCR4 or the CD4 and CCR5 receptors. The NP2 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 4.5g/l glucose, L-glutamine (GIBCO), 5% (v/v) FCS, 1% (v/v) penicillin/streptomycin (complete DMEM) plus the antibiotics, G418 sulphate (100µg/ml, Sigma) for selection of CD4 expression and puromycin (1µg/ml, Sigma) for the selection of CCR5 or CXCR4 co-receptor expression.

The Hela-based cell line (TZM-bl) (Platt et al., 1998), stably transfected with CD4 plus CCR5 and CXCR4 HIV-1 co-receptors, was grown in complete DMEM. This cell line contains the Lac Z gene under the transcriptional control of the HIV-1 LTR which allows identification of infected cells by β-galactosidase (β-gal) staining (Kimpton et al., 1992).

Peripheral blood mononuclear cells (PBMCs) were separated from donor blood (National Blood Service, Tooting, London) and cultured according to the protocol described below.

All cells were incubated at 37°C in an atmosphere of 5% CO₂. Cell viability was determined using 0.4% (w/v) Trypan Blue solution (Sigma).
2.2 Preparation, storage and culture of PBMC’s from donor blood

Buffy coat residues (pooled leukocyte enriched whole blood) were obtained from the National Blood Service. These contained pooled blood from several donors. The blood was diluted 2-fold with sterile phosphate buffered saline (PBS). Diluted blood (20ml) was carefully layered onto equal volumes of Histopaque solution (Sigma-Aldrich) in 50ml centrifuge tubes and centrifuged at 800 x g for 30 minutes without brake. The PBMCs at the Histopaque-plasma interface were removed, transferred to a fresh 50ml tube, resuspended in 45ml of PBS and pelleted by centrifugation at 400 x g for 5 minutes. Repeated washings in PBS were carried out until the supernatant was clear. The washed cells were then either immediately cultured and phytohaemagglutinin (PHA)-stimulated or cryopreserved, as described below.

For the cryopreservation of PBMCs, cells were pelleted by centrifugation at 400 x g for 5 minutes and resuspended at an input density of 3 x 10^7 PBMC/ml in ice-cold cryoprotective medium (50% heat-inactivated FCS, 40% RPMI 1640 with glutamine and 10% DMSO), which was added drop-wise to the cells with constant mixing. The cells were aliquoted into 1ml cryovials (NUNC) and placed in an insulated container, then stored at -80°C, allowing reductions in temperature of 1°C per minute overnight. The following day the cells were stored in liquid nitrogen.

To culture PBMCs, frozen cells were thawed rapidly at 37°C in a water bath and transferred into 25ml of cold complete RPMI then centrifuged at 400 x g for 5 minutes. The cell pellet was resuspended in 10-15ml of complete RPMI medium and transferred to a 25cm^2 tissue culture flask. Cells were stimulated with 1µg/ml of phytohaemagglutinin (PHA) to induce the expression of the IL-2 receptor and incubated at 37°C, 5% CO₂ for 2-5 days before use. After PHA-stimulation, cells were resuspended in medium containing 10 IU IL-2/ml and fed with fresh medium twice weekly. To stimulate cell growth, PBMCs from at least 3 donors were mixed. Viability was determined using Trypan Blue solution, as described in Section 2.1. Only cultures with >80% viability were used for further assays.
2.3 HIV-1 Isolates

HIV-1<sub>MN</sub> (Gallo <i>et al.</i>, 1984) is a B-clade, CXCR4 co-receptor trophic, HIV-1 laboratory-adapted isolate and was propagated using the H9 cell line (Popovic <i>et al.</i>, 1984). The B-clade laboratory-adapted HIV-1 isolate, NL4-3 (Adachi <i>et al.</i>, 1986), is also a CXCR4-trophic isolate and was also propagated using the H9 cell line.

HIV-1<sub>JRCSF</sub> (Koyanagi <i>et al.</i>, 1987) is a B-clade, CCR5 using isolate derived from the cerebral spinal fluid of a patient with AIDS-related dementia and was grown in PBMCs (Koyanagi <i>et al.</i>, 1987).

Clade B patient isolates J98, W49, T01, D71, Y65, C29, X20, H44, M04, Q19, T04 and R04 were expanded from primary culture infected cell supernatant using negative donor PBMC’s.

Primary isolates were selected blindly from a clinical study SPARTAC: a multicentre randomised controlled trial investigating the role of short pulse Antiretroviral therapy in PHI powered to CD4 decline. In this study, primary infection was defined as documented HIV-1 sero-conversion by one or more of the following criteria: (1) positive antibody test within 6 months of a negative test, (2) antibody negative, but proviral PCR or p24 antigen positive, (3) detuned assay with test incidence below 0.6 and/or (4) clinical manifestations of symptomatic HIV-1 sero-conversion illness supported by antigen positivity and by bands on Western blot. The study was performed in accordance with GCP and ethical approval with all patients giving full informed consent. Virus was isolated as described below from blood samples taken at baseline (week 0), defined as the first time-point at which HIV-1 infection was detected within 6 months of sero-conversion, and at a time point one year later.

Virus containing cell supernatants were passed through a 0.45µm filter upon harvest before being stored at -80ºC. Virus containing cell supernatant from all HIV-1 infected cultures was used as the source of virus for further experiments and was never freeze-thawed more than once.
2.4 Monoclonal Antibodies and Sera

The neutralising monoclonal antibodies (MAbs), described in Table 2.1, were used in the HIV-1 neutralisation and antibody binding assays (All antibodies and sera were purchased from NIBSC). For more details of the antibodies used and the epitopes to which they bind, refer to section 1.7.1.

Patient sera consisted of:

- HIV-1 positive serum from a pooled panel of B clade HIV-1 infected individuals and was used for the laboratory strain HIV-1MN.
- Homologous (isolate T02) antisera taken from an HIV-1 infected individual.
- Three heterologous antisera taken from HIV-1 infected individuals.

All sera were heat-treated at 55ºC for 1 hour to inactivate complement proteins while preserving antibody conformation. Heat-treated, uninfected human serum (PAA, The Cell Culture Company) was used as a negative control.

Two MAbs recognising epitopes in HIV-1 p24, EVA 365 and EVA 366 (NIBSC), were used to detect viral protein production of infected NP2 cells in the HIV-1 titration and neutralisation assays described in sections 2.10 and 2.17 (Hinkula et al., 1990).

2.5 Propagation of HIV-1MN

HIV-1MN was propagated in the H9 cell line, whereby 5x10^5 H9 cells in 0.5 ml AIMV medium were incubated with 1ml of HIV-1MN infected cell supernatant (at a TCID_{50}/ml of ~ 1 x 10^5). Approximately 1ml of the mixture was carefully removed after 2-4 hours to avoid disturbing sedimented cells and replaced with fresh complete RPMI. All cultures were incubated at 37ºC in a 5% CO₂ atmosphere. On day 3, cultures were expanded using a 4-fold excess of uninfected H9 cells in a total volume of 6ml. The following day the culture was expanded to 18ml with fresh medium and virus replication allowed to proceed for a further 4 days. The virus-containing supernatant was then harvested, as described above (section 2.3).
Table 2.1: Properties of HIV-1 neutralising antibodies.

<table>
<thead>
<tr>
<th>MAb</th>
<th>Activity</th>
<th>Epitope</th>
<th>gp120 region</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>b12</td>
<td>Neutralises a range of primary and laboratory-adapted isolates. Conformation-dependent</td>
<td>Discontinuous epitope.</td>
<td>CD4 binding site</td>
<td>(Burton et al., 1991; Barbas et al., 1992; Roben et al., 1994)</td>
</tr>
<tr>
<td>2G12</td>
<td>Conformation- and carbohydrate-dependent epitope. Neutralising antibody but only partially neutralises HIV-1MN and has no C-clade activity.</td>
<td>Carbohydrate mannose residues.</td>
<td>Silent face of gp120 outer domain</td>
<td>(Buchacher et al., 1994)</td>
</tr>
<tr>
<td>2F5</td>
<td>Diverse clade neutralisation. Linear epitope, but neutralisation is conformation-dependent.</td>
<td>ELDKWA, gp41 amino acids (662-667).</td>
<td>MPER region of gp41. Lipid binding to viral envelope.</td>
<td>(Muster et al., 1993; Buchacher et al., 1994; Purtscher et al., 1994)</td>
</tr>
<tr>
<td>4E10</td>
<td>Diverse clade neutralisation. Linear epitope, but neutralisation is conformation-dependent.</td>
<td>NWF(D/N)IT, gp41 amino acids (671-676).</td>
<td>MPER region of gp41. Lipid binding to viral envelope.</td>
<td>(Muster et al., 1993; Buchacher et al., 1994)</td>
</tr>
<tr>
<td>4.8D</td>
<td>Neutralises diverse laboratory-adapted and primary HIV-1 isolates. Conformation-dependent and induced by CD4 binding. Binds to co-receptor binding site.</td>
<td>Discontinuous epitope.</td>
<td>Residues spanning C1-5 and V1/V2 regions of gp120. Contacts bridging sheet.</td>
<td>(Thali et al., 1993)</td>
</tr>
<tr>
<td>447-52D</td>
<td>Neutralising for laboratory-adapted B-clade isolates. Weakly neutralising for primary B-clade isolates</td>
<td>Core epitope, GPG, other V3 residues important.</td>
<td>Central and N-terminal regions of V3 loop.</td>
<td>(Gorny et al., 1992; Conley et al., 1994)</td>
</tr>
</tbody>
</table>

Table 2.1 details the monoclonal antibodies used throughout this study. For each antibody, the activity, epitope structure and location within gp120 are described. Original references are also indicated. 1 = amino acid residues of epitopes based on HIV-1 HXB2 reference envelope glycoproteins.
2.6 HIV-1 isolate propagation from primary culture virus supernatant

Initially, 1ml of plasma from HIV-1 infected individuals was transferred to a 24-well tissue culture plate (NUNC) with 1ml of complete RPMI containing 2 x 10^6 donor PBMCs (PHA-stimulated). A negative control of 2 x 10^6 PHA-stimulated donor PBMC’s in 2ml of complete RPMI medium containing 10 IU IL-2 was also included. The cells were incubated at 37ºC in an atmosphere of 5% CO₂. The next day, 0.5-1ml of culture medium was replaced with fresh medium. On day 7, a small aliquot was assayed for HIV-1 RT activity (assay described below, section 2.12). If positive (RT activity 5 times above background and above 1 μU per 5μl), the culture was expanded into a 25cm² tissue culture flask in a total volume of 5ml containing 4 x 10^6 freshly PHA-stimulated donor PBMC’s. On day 10, half the culture medium was harvested, stored and replaced with complete RPMI medium containing 10 IU IL-2. Cultures were again expanded, on day 14, into a total of 10ml of medium containing 8 x 10^6 freshly PHA-stimulated donor PBMC’s. On day 17, culture supernatant was harvested and replaced as on day 10. The final harvest (day 21) produced 10 x 1ml aliquots of culture supernatant. If higher titres of virus were required secondary cultures were propagated by initially incubating 2x10^6 HIV-1 infected cells from the primary culture with 2x10^6 negative donor cells. These secondary cultures were then treated using an identical method to that described for the primary cultures with respect to expanding and harvesting the HIV-1 isolates.

2.7 Pseudotyped B-clade primary isolate (T02) production

Clade B, primary isolate pseudovirions were generated as previously described by Derdeyn et al, 2004. Briefly, the full length env and rev sequence of a B-clade, primary patient isolate (T02) were cloned into a pcDNA3.1 TOPO-TA vector. Pseudovirions were subsequently produced by co-transfection of 293T cells with the patient env/rev plasmid (pcDNA3.1 TOPO-TA) and env-deficient subtype B proviral plasmid vector, pHXB2ΔEnv, using Fugene-6 (Hoffman-La Roche) according to manufacturer’s instructions. Transfection medium was replaced with complete DMEM on the following day and virus harvested 48 hours later and stored as described above (page 94).
2.8 Cholesterol depletion

A stock solution of 100mM Methyl-β-cyclodextrin (MBCD, Sigma) was prepared in PBS and stored at 4°C for no longer than 1 month. The HIV-1MN isolate, at a titre of 1 x 10^5 TCID₅₀/ml, was incubated with varying concentrations of MBCD (0-12.5mM, detailed in results) at 37°C for 1 hour. Modified virus was purified by ultracentrifugation through a 20% sucrose cushion at 100,000 x g, for 2 hours, in a Sorvall pro80 Ultracentrifuge at 4°C. Viral pellets were resuspended in PBS and stored at -80°C.

2.9 Cholesterol measurement

Cholesterol concentration was determined using the Amplex Red Cholesterol Kit (Invitrogen, Molecular Probes). Briefly, 5mls of HIV-1MN were treated with MBCD, as above. The virus was purified through a 20% sucrose cushion at 100,000 x g, at 4°C for 2.5 hours (Sorvall pro80 Ultracentrifuge) before resuspension in 50µl of Amplex Red buffer (supplied with the kit). Equal volumes of virus (diluted 1/2 and 1/10 with Amplex Red buffer) and Amplex Red reagent (made according to the manufacturer’s instructions with the reagents supplied) were mixed and incubated at 37°C for 1 hour. A dilution series of the cholesterol standard provided was included to construct a standard curve. Uninfected cell supernatant was treated with MBCD in the same way to control for background cholesterol. Fluorescence was measured at 640nm.

2.10 HIV-1 titration

NP2 cells were grown to approximately 75% confluence, before 2 x 10⁴ cells in a volume of 250µl were plated onto a 48-well tissue culture plate (NUNC) in complete DMEM medium and incubated overnight at 37°C in an atmosphere of 5% CO₂. On the following day, virus stock was diluted 10-fold in complete DMEM. The medium was removed from the cells, 100µl of each virus dilution added in triplicate and incubated for 2 hours. Cells were then washed twice with complete DMEM before adding 500µl of complete DMEM and incubated for 48-72 hours at 37°C, in an atmosphere of 5% CO₂. Medium was removed, the cells washed once in PBS and fixed with 250µl/well of ice-cold acetone: methanol (1:1v/v) for 5 minutes. The acetone:methanol mixture was removed and the cells allowed to air-dry before rinsing with 500µl/well of PBS. To the
fixed cells, 100µl of pooled anti-p24 monoclonal antibodies (EVA 365 and EVA 366), see Monoclonal Antibodies and Sera section), diluted to 1:40 with PBS supplemented with 1% FCS, was added and incubated at room temperature for 1 hour. After two more washes with PBS, 250µl of goat anti-mouse IgG1-β-gal conjugated secondary antibody (diluted 1:400 in PBS with 1% FBS) were added and the cells incubated at room temperature for 1 hour. After a further two washes with PBS (500µl/well), 250µl/well of β-gal substrate (3mM K₄Fe(CN)₆, 1mM MgCl₂ and X-gal (1-0.4mg/ml) in PBS) were added and the cells incubated at 37°C for 3-5 hours. Foci of infection (assayed as Focus Forming Units, FFU), stained blue, were counted to estimate virus titre.

2.11 Syncytium induction assay

A total of 2 x 10⁵ C8166 cells in 0.5ml complete RPMI medium were added to each well of a 24-well flat bottomed tissue culture plate (NUNC). Virus was diluted in 10-fold dilutions in complete RPMI medium, and 0.5ml of each dilution, in duplicate, was added to the cells. The cells were incubated for 1 hour at 37°C in an atmosphere of 5% CO₂. Subsequently, 1ml of complete RPMI was added and the cells returned to the incubator and observed over 5-7 days for the presence of syncytia. To calculate viral titre, cultures were scored positive if more than 5 syncytia were present and the TCID₅₀/ml of the virus was calculated using the Spearman-Karber method.

2.12 HIV-1 Reverse Transcriptase Activity

Reverse Transcriptase activity was determined by quantitative reverse transcriptase PCR (qRT-PCR) using an RNA template derived from brome mosaic virus (BMV) (Promega) (Pizzato et al., 2008). A total volume of 5µl of virus was mixed with an equal volume of lysis buffer (100mM Tris-HCL, pH 7.4, 50mM KCl, 0.25% (v/v) Triton X-100, 40% (v/v) glycerol and 0.4U/µl RNAse inhibitor, pH7.5) and incubated at room temperature for 5 minutes. These samples were diluted 10-fold using dilution buffer (20mM Tris-HCL, 20mM KCL and 5mM (NH₄)₂SO₄, pH8.3), a 5µl aliquot added to LightCycler capillary tubes (Roche) and mixed gently with 5µl of reaction buffer containing 20mM Tris-HCL, 20mM KCL, 5mM (NH₄)₂SO₄, 200µM dNTPs, 0.5µM forward and reverse primers (Forward primer, 5’-
CGTGGTTGACACCAGACCTCTTTAC-3’ and reverse primer, 5’-GGTCTCTTTTAGATTACAGTG-3’), bovine serum albumin (BSA) 0.1µg/ml, 1/20000 SYBR green I, 12ng BMV template RNA and 0.2U Hotstart Taq polymerase (MBI Fermentas). Thermal cycler (Lightcycler 2.0, Roche) conditions were as follows: Reverse transcription at 37°C for 30 minutes, denaturation at 95°C for 5 minutes, followed by 45 cycles of amplification at 95° for 5 seconds, 55°C for 5 seconds, 72°C for 15 seconds and 83°C for 7 seconds. Recombinant RT (ARP 631.1, NIBSC) was diluted in dilution buffer and used to make a standard curve.

2.13 HIV-1 p24 ELISA

A 96-well plate (NUNC Maxisorp) was coated overnight at room temperature with 100µl per well of anti-p24 monoclonal antibody, D7320 (Aalto Bioreagents, Dublin) at 10µg/ml in 100mM NaHCO₃ buffer, pH 8.5. Each well was subsequently washed twice with 200µl of Tris-buffered saline (TBS, 144mM NaCl, 25mM Tris at pH7.5). All infected cell supernatants were pre-treated with 1% Empigen BB (Sigma), disrupting virions to release the p24 protein, and heat-inactivated at 56°C for 1 hour to allow removal from the Category 3 facility. Infected cell supernatants (100µl/well) were added along with a standard curve constructed by two-fold dilution of stock p24 (Aalto Bioreagents, Dublin) in TBS with 0.05% Empigen BB to give a range of dilutions from 10ng/ml to 40pg/ml. Non-specific background readings were obtained by adding 100µl of uninfected cell supernatant treated with 1% Empigen BB and heat-inactivated, as above. The cell supernatants were incubated for 3 hours at room temperature. After washing twice with 200µl/well of TBS, anti-p24 alkaline phosphatase (AP)-conjugated monoclonal antibody (Aalto) was diluted 1:2500 with TBS containing 2% (w/v) skimmed milk powder, 20% (w/v) BSA and 0.05% (v/v) Tween-20. This detection antibody was added at a volume of 100µl/well and incubated at room temperature for 1 hour. The AMPAK amplification system (Dako Ltd) was used to visualise p24, as described in the manufacturer’s instructions. Absorbance was read at 492nm. A cut-off value calculated from the average background readings, assayed in triplicate, plus two times standard deviation.
2.14 HIV-1 gp120 ELISA

A stock solution of 15µg/ml of the anti-gp120 polyclonal antibody, D7324 (Aalto Bioreagents, Dublin) was made in PBS buffer, pH 6.5, and used to coat a 96-well plate (NUNC Maxisorp) overnight at 4°C, with 50µl per well of the antibody solution. All wells were washed three times with 200µl of Wash Buffer (WB), PBS, pH7.4 and 0.05% (v/v) Tween-20 and non-specific binding sites were blocked for 1 hour at room temperature with 100µl/well of Blocking Buffer (BB, PBS, pH7.4 containing 0.05% (v/v) Tween-20 and 5% (v/v) porcine serum). A standard curve was constructed by diluting a stock solution of recombinant HIV-1 MN gp120 (NIBSC) in BB to 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001ng/ml. After washing, as above, 100µl/well of infected cell supernatant dilutions and standards were added and incubated for 2 hours at 37°C. Negative controls consisted of BB alone and HIV-1 negative PBMC supernatant. The wells were washed, as above, before 50µl/well of HIV-1 positive IgG antisera (ARP 509, NIBSC) diluted to 1:500 with BB was incubated for 1 hour at room temperature. Following three further washes, the secondary detection antibody, anti-human IgG-AP conjugate, was diluted 1:5000 with BB and 50µl added to each well before incubation at room temperature for 1 hour. After three final washes, gp120 was visualised using tetramethyl benzidine (TMB) reagent (BioRad), as described in the manufacturer’s instructions. Absorbance was read at 492nm (Moore et al., 1988). A cut-off value was calculated from the average of the negative controls plus two times the standard deviation.

2.15 Western blotting

HIV-1 infected cell supernatant dilutions or control uninfected cell supernatant were mixed with 2 x Laemmli buffer (Laemmli, 1970) and boiled at 95°C for 5 minutes. Proteins were resolved by SDS polyacrylamide electrophoresis using 4-20% Tris-glycine gels (NOVEX) at 125V for 1.5 hours using buffers recommended by the manufacturer. Samples were blotted onto nitrocellulose membrane by semi-dry transfer at 160mV for 1.5 hours and non-specific protein binding sites blocked with PBS containing 0.05% Tween-20 (PBST) and 5% non-fat milk powder, at 4º overnight (o/n). To visualise envelope glycoprotein gp120 the membrane was incubated with rabbit anti-
gp120 polyclonal serum diluted 1:2000 (ARP421, NIBSC) for 1 hour at room temperature before a series of three 15 minute washes in PBST at room temperature. An anti-rabbit HRP-conjugated secondary antibody (Sigma) was then incubated with the membrane for 1 hour at room temperature at a dilution of 1:2000. Following three further 15-minute washes, protein bands were visualised using chemiluminescence as per manufacturer’s instructions (ECL plus, Amersham). Blots were exposed onto X-Omat AR film (KODAK) and developed.

The Western blot protocol above was carried out to visualise all HIV-1 proteins. A murine anti-p24 monoclonal antibody (ARP 432, NIBSC) diluted 1:2000 together with anti-murine HRP-conjugated secondary antibody (Sigma), also diluted 1:2000, probed for HIV-1 p24. To identify gp41, mouse-monoclonal anti-gp41 produced by a hybridoma cell line (NIH) was used at a dilution of 1:50 followed by anti-murine HRP-conjugated secondary antibody at 1:1000. Pooled patient HIV-1 positive sera (section 2.4), diluted 1:100 and combined with an anti-human HRP-conjugated secondary antibody (1:2000 dilution) was used to detect a wider range of HIV-1 proteins.

2.16 Transmission Electron Microscopy

A 5µl drop of concentrated virus (3mls of treated virus at \(10^5\) TCID_{50}/ml pelleted through a 20% sucrose cushion and resuspended in 25µl of PBS containing 0.5% gluteraldehyde) was placed onto parafilm and a charged electron microscope grid was placed on the drop for 5 minutes. The grid was subsequently washed 5 times with PBS and incubated on a small drop of 1% (w/v) uranyl acetate for 30 seconds before air-drying. Once dry, the grid was examined by transmission electron microscope (Technai G2, FEI Nanotech) at x 43,000 magnification (Dourmashkin et al, 1993). This process was repeated for all concentrations of MBCD-treated virus.

2.17 HIV-1 Neutralisation assay

Neutralisation of virus grown in H9 cells, HIV-1\textsubscript{MN}, was assessed by NP2 cells. Cells were grown to approximately 75% confluence, before 250µl of cells (2 x 10^4 cells/well) were plated onto a 48-well tissue culture plate (NUNC) in complete DMEM medium and incubated overnight. Cells were checked for 50-75% confluence and virus was
diluted to 150 FFU/well. Virus diluted to 150 FFU/well was then pre-incubated at 37°C for 1 hour with increasing concentrations of neutralising antibody (detailed in Results – 0-2µg/ml), in a total volume of 100µl/well in triplicate. The negative control consisted of virus incubated with HIV negative sera (1:10). Once cells had reached 50-75% confluence, the medium was removed and 100µl of virus/antibody mixture was added per well in triplicate and incubated for 2 hours at 37°C. The inoculum was removed and cells were washed twice with DMEM with 5% FCS before addition of 500µl of complete DMEM and incubation for 48 hours at 37°C in an atmosphere of 5% CO₂. After removing the medium, cells were washed once in PBS and fixed with 250µl/well of ice-cold acetone: methanol (1:1) for 3-5 minutes. The acetone:methanol mixture was removed and the cells air-dried before rinsing with 500µl/well of PBS.

A stock solution of pooled anti-p24 monoclonal antibodies (EVA 365 and EVA 366 from NIBSC) was made at a dilution of 1:40 with PBS (antibody:PBS) and supplemented with 1% FCS. To each well, 100µl of this stock solution was added before incubation at room temperature for 1 hour. After two further washes with PBS, 250µl of goat anti-mouse IgG1 β-gal conjugated secondary antibody diluted 1:400 in PBS with 1% FCS was added and the cells were incubated at room temperature for 1 hour. After washing twice with PBS (500µl/well), 250µl/well of β-gal substrate (3mM K₄Fe(CN)₆, 1mM MgCl₂ and X-gal (1mg/ml) in PBS), the cells were incubated at 37°C for 3-5 hours. Foci of infection, stained blue, were counted to estimate virus neutralisation. Percentage infectivity inhibition was calculated against virus incubated with HIV-1 negative serum.

2.18 Estimation of HIV-1 MAb binding by immunoprecipitation

A 96-well plate (NUNC Maxisorp) was coated overnight at 4°C with 100µl per well of monoclonal antibodies 2F5 (2µg/ml), 2G12 (10µg/ml), b12 (1µg/ml), 4E10 (2µg/ml) and 4.8D (2µg/ml) in carbonate buffer (15mM Na₂CO₃, 35mM NaHCO₃ and 3 mM NaN₃, pH 9.6). The plate was subsequently washed three times with PBS, 200µl per well, to remove unbound antibody. Non-specific binding sites were blocked for 4 hours at 4°C with PBS containing 5% (w/v) pig serum (Sigma). Virus containing 150µU of RT activity in 100µl of complete RPMI medium were added and incubated overnight at
4°C. Negative controls consisted of uninfected cell supernatant with or without MBCD treatment to remove cholesterol. To ensure specificity of binding, ΔenvNL4-3 virus was included as a control along with viral samples spiked with 0.5μg/ml of recombinant HIV-1 IIIB gp120 (EVA607, NIBSC). Unbound virus was removed from the wells by 5 washes in PBS (200μl/well), before the addition of 110μl lysis buffer (1% Triton X-100 in PBS) to each well. The plate was shaken, in a shaking incubator, for 15 minutes at room temperature before MAb bound virus was quantified by p24 content, described in Section 2.13.

To obtain higher sensitivity within the antibody binding assay, bound virus was also quantified by RT activity (see HIV-1 Reverse Transcriptase Activity method, section 2.12). The method used was identical to that described above except the lysis buffer was identical to that described in Section 2.12.

2.19 Virus isolation for virion bound gp120 quantification

HIV-1 infected-cell supernatant was filtered through a 45μm filter (Millipore) to remove cellular debris. Virus from 5ml of infected-cell supernatant was purified by ultracentrifugation (Beckman Optima™ TLX Ultracentrifuge) through a 20% sucrose cushion at 100,000 x g for 2 hours to isolate intact virions. The pellet was resuspended in PBS and RT activity and gp120 content quantified in the assays described above (section 2.12 and 2.14, respectively). The uninfected cell supernatant control was treated in exactly the same as the virus samples.

2.20 Proviral DNA extraction

Proviral DNA was extracted from PBMC’s taken from primary cultures of four HIV-1 isolates (X20, C29, J98 and M04) using the QIAampDNA blood mini kit (Qiagen). The PBMC’s (5 x 10^6) were washed once with PBS, pelleted by centrifugation at 10,000 x g (all subsequent centrifugation was also carried out at this speed) and resuspended into 200μl of PBS. Proteinase K (20μl) was added to the cell suspension to inactivate nucleases that might degrade the genomic DNA in the subsequent purification steps. The PMBC’s were lysed with 200μl of buffer AL and incubated at 56°C for 10 minutes to maximise DNA yield. Absolute ethanol (200μl) was added to the lysate to precipitate
the genomic DNA. The resulting mixture was transferred into a spin column to bind DNA to the membrane column and centrifuged for 1 minute, while contaminants were eluted and discarded. Residual contaminants were removed with one wash of buffer AW1 (500µl) followed by another wash with buffer AW2 (500µl) with centrifugation for 1 minute each time. The flow-through was discarded and any residual wash buffer removed by further centrifugation for 1 minute. Bound DNA was eluted by addition of 100-150µl of buffer AE (10mM Tris-HCl, 0.5mM EDTA, pH9.0) to the membrane, followed by centrifugation for 1 minute. The eluted DNA was diluted 1:50 in distilled water and its concentration calculated by absorbance (at 260nm) using the appropriate program on a Ultraspec 2000 spectrophotometer (Pharmacia Biotech) and corrected for the dilution factor of 50.

2.21 Amplification of primary isolate gp41 sequences.

Proviral DNA extracted from infected PBMC’s (section 2.20) constituted the template for polymerase chain reaction (PCR) amplification of N-terminus of the gp41 env gene. This was done using the forward and reverse primers, 5’-GCCTTAGGATCTCTCCTATGCGAGGAAGAA-3’ and 5’-TTGTAAGTCTGTTACTAAAGGTAC-3’, respectively. Easy-A High Fidelity polymerase (Strategene) was employed for all PCR amplifications with the reaction conditions: 1-X Easy A buffer with 1.5mM MgCl₂, 20pmol of each primer, 0.25mM dNTP’s and enough double-distilled water to make a final reaction volume of 50µl. Generally, between 100ng to 1µg of total DNA served as a template for the PCR amplification. The cycling parameters were as follows: 1 cycle of hotstart polymerase activation at 95°C for 2 minutes, 35 cycles of amplification at 95°C (15 seconds), 55°C (15 seconds) and 72°C (4 minutes), and 1 cycle of final elongation at 72°C for 7 minutes. The PCR product was separated on a 1% agarose gel by electrophoresis at 120V for 40 minutes and was approximately 700bp in size. The DNA was purified away from non-specific products by gel extraction using the QIAquick gel extraction kit (Qiagen).
2.22 Purification of amplified gp41 sequences by gel extraction

To extract the DNA from a gel, the required band was excised from the agarose with minimal exposure to ultraviolet light and weighed. A volume of buffer QG (Qiagen) 3 times the weight of the gel band was added, and the mixture was solubilised at 50°C for 10 minutes. To precipitate the DNA, 1 volume of isopropanol was added to the mixture. The resulting solution was centrifuged at 10,000 x g (all subsequent centrifugation was also carried out at this speed) for 1 minute in a QIAquick spin column, during which time the DNA was bound to the column membrane and small contaminants were eluted and discarded. The column was then washed with 750µl of buffer PE by centrifugation for 1 minute. The flow-through was discarded and any residual wash buffer removed by a further centrifugation for 1 minute. Bound DNA was eluted by addition of 30µl of buffer EB (10mM Tris-HCl, pH8.5) to the membrane, followed by centrifugation for 1 minute.

2.23 Sequence analysis of primary isolate gp41

Amplified gp41 DNA sequences from the four primary isolates were used for sequence analysis. The sequencing reaction was prepared by mixing 200-500ng of the plasmid DNA with 3.2pmol of the primer and enough water to bring the final volume to 10µl. The gp120 sequencing mixtures were sent to the Genomic Core Laboratory (Medical Research Council, Imperial College) for analysis. All returned data was analysed with BioEdit software. The DNA sequences were translated and located on the HIV-1 genome using the sequence locator tool from the Los Alamos HIV-1 database website (www.hiv.lanl.gov) and aligned with other primary and laboratory-adapted HIV-1 env amino acid sequences using the Multalign online server (http://bioinfo.genotoul.fr/multalin/multalin.html).

2.24 HIV-1 neutralisation assay using TZM cells

Viral neutralisation of the primary pseudotyped isolate, T02, was assessed by TZM cells. Complete DMEM medium containing 2 x 10^4 TZM cells in 400µl was seeded into each well of a 48-well plate and incubated overnight or until they reached 50% confluence. Virus (diluted to 150 FFU/well) was pre-incubated at 37°C for 1 hour with
neutralising antibodies in increasing concentrations (detailed in results, 0-2µg/ml) in a total volume of 150µl/well. The negative control consisted of virus incubated with HIV negative serum (1:10). The medium was removed from the cells, and 150µl of virus/antibody mixture was added, in triplicate, before being incubated for 2 hours at 37°C. The total volume of complete DMEM per well was then increased to 500µl and incubated for 48 hours at 37°C in an atmosphere of 5% CO₂. The medium was removed and the cells fixed with 0.5% gluteraldehyde in PBS for 10 minutes at room temperature. After washing twice with PBS (500µl/well), 250µl/well of β-gal substrate (3mM K₄Fe(CN)₆, 1mM MgCl₂ and X-gal (1mg/ml) in PBS) was added and incubated with the cells at 37°C for 3-5 hours. The TZM cell line contains the Lac Z gene under transcriptional control of HIV-1 LTR to allow identification of infected cells by β-gal staining. Foci of infection (Focus forming units, FFU), stained blue, were counted to estimate virus neutralisation. Percentage infectivity inhibition was calculated against virus incubated with HIV-1 negative serum.

2.25 Virus inactivation

For viral inactivation using formalin (Sigma) and heat inactivation, formaldehyde was added to HIV-1MN (infected cell supernatant) at a final concentration of 0.02% (v/v) and incubated for 30 minutes at room temperature. Any residual formaldehyde was quenched with 0.2% BSA in PBS. Virus supernatants were then heat-inactivated at 45°C or 62°C for 30 minutes in a heating block (Grovit-Ferbas et al., 2000; Poon et al., 2005; Poon et al., 2005).

To inactivate virus using Aldrithiol-2 (AT-2, Sigma), a stock solution of 100mM AT-2 in DMSO was freshly prepared and added to HIV-1MN samples at a final concentration of 1mM. Viral samples containing 1mM AT-2 were incubated at 37°C for 1 hour (Arthur et al., 1998).

N-ethylmaleimide (NEM) was incubated with viral samples at a final concentration of 1mM for 1 hour at 37°C. Following this inactivation stage, unreacted NEM solution was quenched with excess reduced (Morcock et al., 2005) glutathione and 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer (pH 7.5).
For β-propiolactone (BPL, Sigma) inactivation of HIV-1, a final concentration of 0.2% β-propiolactone, diluted in PBS, was incubated with the virus for 22 hours at room temperature (Race et al., 1995; Race et al., 1995).

The chemical structure of all the above inactivating reagents is illustrated in Figure 2.1. All inactivating agents were removed by ultrafiltration through a 300kDa cut-off device (Sartorius) at 2000 x g for 15 minutes. To combine cholesterol-depletion with inactivation, virus was first incubated with 1mM MBCD before inactivation. The virus was then resuspended in PBS and stored at -80ºC. Uninfected cell supernatant, as the negative control, was processed in the same way.

2.26 PBMC titration

Initially, virus was diluted 5-fold (1/5 to 1/625) in complete RPMI containing 10 IU IL-2/ml. To a 96-well tissue culture plate (NUNC), 75µl of the virus dilutions was added in triplicate. To all wells, 150µl of PHA-stimulated mixed donor PBMCs, at 1 x 10^5 cells/ml, were added. Cells and virus were incubated at 37ºC in 5% CO₂. On days 2, 3 and 4, cells were washed with 180µl of complete RPMI containing 10 IU IL-2/ml to remove the virus inoculum. Care was taken not to disturb or remove sedimented cells. On day 7, 150µl was removed from each well for p24 antigen estimation using the HIV-1 p24 ELISA (section 2.13). The medium taken for p24 testing was replaced with fresh complete RPMI containing 10 IU IL-2/ml. Sampling of p24 was carried out, as described above, weekly for 3 weeks.

2.27 HIV-1 infectivity assay

A total of 2 x 10^5 C8166 cells in 150ml complete RPMI were added to each well of a 96-well flat bottomed tissue culture plate (NUNC). Virus was diluted in 5-fold dilutions in complete RPMI medium, and 50µl of each dilution, in triplicate, was added to the cells. The cells were observed over 5-7 days for cytopathic effect in the form of multi-nucleated cells (syncytia) (Chatterjee et al., 1980; Weiss et al., 1986; Timar et al., 1987). The extent of the virus-induced cytopathic effect was scored depending on the
Figure 2.1. HIV-1 inactivating reagents

FORMALDEHYDE  β-PROPIOLACTONE

ALDRITHIOL-2  N-ETHYLMALEIMIDE

Figure 2.1. Chemical structure of the 4 reagents used to inactivate HIV-1MN.
number of syncytia present (+ = more than 5 syncytia, ++ = more than 15 and +++ =
more than 25).

2.28 Estimation of inactivated HIV-1 MAb binding by ELISA

A 96-well plate (NUNC Maxisorp) was coated overnight at 4°C with 100µl per well of
MAb b12 (1µg/ml) in carbonate buffer (15mM Na₂CO₃, 35mM NaHCO₃ and 3 mM
NaN₃, pH 9.6). The next day, the plate was washed (200µl/well) three times with PBS
to remove unbound antibody. Non-specific binding sites were blocked for 4 hours at
4°C with PBS containing 5% (w/v) pig serum (Sigma). Samples consisting of 100ng of
p24 in 100µl of complete RPMI were added and incubated overnight at 4°C. Negative
controls consisted of uninfected cell supernatant with or without MBCD treatment.
Unbound virus was washed from the plate with PBS (5 x 200µl/well washes), before the
addition of 100µl of lysis buffer (section 2.13). The lysis buffer was incubated in the
wells for 15 minutes at room temperature in a shaking incubator. The MAb bound virus
was then quantified using the HIV-1 RT Activity method described (section 2.12), or by
HIV-1 p24 ELISA (section 2.13).

2.29 HIV-1 Immunogens for in vivo immunisation

A total of 250µl of concentrated (1mg/ml total protein) AT-2 inactivated HIV-1MN
(supplied by NIH), grown in the SupT1 cell line, was diluted in 10ml of sterile PBS
containing 1mM MBCD and incubated at 37°C for 1 hour. Untreated control virus was
incubated in PBS alone. Negative control immunogens consisted of matched SupT1
uninfected cell supernatant (supplied by NIH) were prepared in the same way as the
virus, with and without MBCD treatment. Virus was then purified by ultracentrifugation through a 20% sucrose cushion at 100,000 x g at 4°C for 2 hours in a
Sorvall Ultra Pro8 Ultracentrifuge. Virus pellets were resuspended in 300µl of sterile
PBS and stored at -150°C. Viral protein (gp120, gp41 and p24) and cholesterol content
were later assayed by ELISA and Western blot (sections 2.13 – 2.15). The protein
content of the viral preparations is detailed in Chapter 6. Ablation of infectivity was
tested by titration of virus onto PMBC and C8166 cells (sections 2.10 and 2.11,
respectively). Immunogens were mixed with an equal volume of Titermax Gold
adjuvant (Invitrogen), as per manufacturer’s instructions and 100µl of the mixture dispensed into 1ml syringes, with 26 gauge needles attached, ready for inoculation. The immunisation is detailed in Figure 2.2.

2.30 Antibody response ELISA

Serum samples taken on Day 55 (after all three immunisations) were assayed for protein-specific antibody titres by ELISA. A 96-well plate (NUNC Maxisorp) was coated overnight at 4°C with 50µl per well of HIV-1 antigens in carbonate buffer (100mM NaHCO₃, pH 8.5). Antigens included recombinant HIV-1_HIV gp120 at 5µg/ml (EVA607, NIBSC), recombinant p24 at 5µg/ml (D7324, Aalto Bioreagents, Dublin), recombinant C-clade ZM96 gp140 at 5µg/ml (gift of Dr Simon Jeffs, Imperial College London) and AT-2 inactivated HIV-1-MN (10µg/ml total protein, NIH) disrupted using 0.5% Triton X-100 to release viral antigens. The wells were washed (200µl per well) three times with wash buffer (PBS containing 0.05% Tween-20) to remove unbound antigen. Non-specific protein binding sites were blocked for 1 hour at room temperature with PBS containing 2% (w/v) pig serum (Sigma). Sequential 2-fold serum dilutions from 1 in 100 to 1 in 12,800 (total volume of 50µl) were added to wells along with negative and positive controls and incubated at room temperature for 1 hour. The negative control consisted of pre-bleed sera pooled from 5 mice, diluted 1/100. The positive control serum originated from mice immunised with recombinant HIV-1_BAL gp120 (gift of Professor Quentin Sattentau, University of Oxford) and was diluted 1/100. After washing away unbound antibodies, as previously described, the wells were incubated with 50µl of secondary anti-mouse conjugated to HRP, diluted 1/1000 in blocking buffer, for 1 hour at room temperature. After a final series of three washes, as described, and an additional wash (200µl/well) of distilled water to remove traces of Tween-20 that may affect the substrate reaction, the plate was developed using TMB substrate (BioRad), according to the manufacturer’s instructions. Colour development was allowed to continue for 5-10 minutes and the reaction stopped using 2.5M H₂SO₄. Absorbance was recorded at 450nm. A cut-off value to determine true negative samples were calculated from the average of triplicate pre-bleed serum samples (diluted 1 in 100 with PBS containing 2% pig serum) plus two times standard deviation.
Figure 2.2: Mouse immunisation schedule.

Figure 2.2 Vaccination schedule. See section 2.29 for details.
2.31 Neutralising antibody responses

Neutralising antibody responses were analysed using the TZM neutralisation assay, described in section 2.24, adapted to a 96-well plate. Briefly, HIV-1MN stocks were diluted to 150 Focus Forming Units (FFU)/well and pre-incubated with 100µl of mouse sera at a 1 in 50 dilution for 1 hour at room temperature with agitation. Virus and serum mixtures were added to triplicate wells of a 96-well flat-bottomed tissue culture plate (NUNC) and 1 x 10⁴ cells in 100µl of complete DMEM added. This resulted in a final serum concentration of 1 in 100. The plate was incubated at 37°C for 2 hours in 5% CO₂. The negative control was virus incubated with prebleed sera, pooled from 5 mice (1:100 dilution) and the positive control consisted of virus incubated with pooled HIV-1 antisera (diluted 1:500) combined with 1µg/ml 2F5 antibody. Medium was removed from the cells 2 hours later and replaced with 200µl complete DMEM before incubation for a further 48 hours. Following this medium was removed and cells fixed with 0.5% glutaraldehyde in PBS for 10 minutes at room temperature. After washing twice with PBS (200µl/well), 150µl of β-gal substrate (3mM K₄Fe(CN)₆, 1mM MgCl₂ and X-gal (1mg/ml) in PBS) was added to all wells with incubation at 37°C for 3-5 hours. The TZM cell line contains the Lac Z gene under transcriptional control of HIV-1 LTR to allow identification infected cells by β-gal staining. FFU, stained blue, were counted to estimate virus neutralisation. Percentage infectivity inhibition was calculated against the negative control.

2.32 Statistical analysis

All statistical analysis was done using the SPSS microsoft program. Significance values were calculated by the non-parametric Mann-Whitney test using a 95% confidence interval. Where multiple comparisons were made the confidence interval was increased according to Bonferroni’s correction.
Chapter 3:

Cholesterol depletion of the HIV-1$_{MN}$ envelope enhances gp160 antigenicity.
3.1 Introduction

The HIV-1 envelope is lipid raft-like in composition (Aloia et al., 1988; Aloia et al., 1993), whereby the levels of PC and PI are reduced by at least 50%, but cholesterol and GSL’s are present in up to 3-fold higher concentrations than those found in cellular membranes (Aloia et al., 1993). This lipid composition is important for viral replication as an increase in PC within the viral envelope, which reduces the membrane lipid raft-like composition, has been shown to decrease viral infectivity (van Til et al., 2008).

Cholesterol is important during HIV-1 replication at a number of stages (Liao et al., 2001; Guyader et al., 2002; Popik et al., 2002; Viard et al., 2002; Jolly et al., 2005). Not only is it an essential component of the virus envelope, facilitating fusion with the target cell (Guyader et al., 2002; Carter et al., 2009), but its removal from cells before infection also inhibits virus-cell fusion (Liao et al., 2001; Popik et al., 2002; Viard et al., 2002). Moreover, cholesterol has been shown to be important in virological synapse formation and transmission of HIV-1 directly between cells (Jolly et al., 2005).

Much of the work exploring the role of cholesterol in HIV-1 biology has been carried out using cholesterol-modifying compounds, such as the β-cyclodextrins (Pitha et al., 1988; Manes et al., 2000; Nguyen et al., 2000; Alfsen et al., 2001; Campbell et al., 2002; Guyader et al., 2002; Nguyen et al., 2002; Popik et al., 2002; Viard et al., 2002; Argyris et al., 2003; Graham et al., 2003; Jolly et al., 2005; Rawat et al., 2005; Ablan et al., 2006; Rawat et al., 2006; Ono et al., 2007). The β-cyclodextrins are a group of ring-structured sugars that bind cholesterol (cholesterol chelators) and have been widely used to extract cholesterol from the HIV-1 envelope (Pitha et al., 1988; Liao et al., 2001; Campbell et al., 2002; Guyader et al., 2002; Popik et al., 2002; Viard et al., 2002; Graham et al., 2003; Liao et al., 2003; Rawat et al., 2005; Rawat et al., 2006; Ono et al., 2007). In particular, MBCD depletion of viral envelope cholesterol has helped to define the role of lipid rafts in HIV-1 fusion, the virological synapse and viral budding (Manes et al., 2000; Nguyen et al., 2000; Campbell et al., 2002; Guyader et al., 2002; Nguyen et al., 2002; Popik et al., 2002; Viard et al., 2002; Argyris et al., 2003; Graham et al., 2003; Jolly et al., 2005; Ono et al., 2007). The role of cholesterol in the HIV-1 life cycle is discussed in more detail in Section 1.6.
Lipid-depleted SIV<sub>mac</sub> has been shown to enhance the immunogenicity of inactivated SIV<sub>mac</sub> <em>in vivo</em> (Kitabwalla <em>et al.</em>, 2005; Kitabwalla <em>et al.</em>, 2008). Although never investigated, this suggests that cholesterol depletion of the HIV-1 envelope by MBCD may affect protein presentation at the viral surface. It follows, therefore, that cholesterol-modifying compounds may be useful for manipulating viral immunogens in order to increase immunogenicity. The aim of this chapter was to investigate the effect of removing cholesterol from HIV-1 on virus infectivity, protein composition, virus morphology and HIV-1 Env antigenicity.
3.2 Results

The effect of MBCD treatment on viral envelope cholesterol and infectivity.

In order to remove cholesterol from HIV-1\textsubscript{MN}, supernatant fluid harvested from H9 cells chronically infected with HIV-1\textsubscript{MN} was incubated with MBCD in a range of concentrations (1-12.5mM). Figure 3.1 shows that cholesterol was significantly removed from the viral envelope and that depletion followed a biphasic pattern. Initially, a plateau was observed, indicating slow cholesterol removal up to 0.5mM MBCD, followed by a sharp drop in cholesterol levels between 0.5 to 2.5mM MBCD. Thereafter, at 5mM concentrations and above, only small reductions in envelope cholesterol occurred, as viral cholesterol content was minimal (<5%).

Removing cholesterol from HIV-1\textsubscript{MN} had a profound effect on virus infectivity. The infectivity of native and cholesterol-depleted HIV-1\textsubscript{MN} was determined by titrating virus on NP2 cells. Infected cells were visualised by internal p24 staining and the percentage infectivity was calculated against virus incubated in PBS without MBCD (untreated control). Estimation of viral infectivity post-treatment (Figure 3.2) indicated that at low concentrations (1mM) of MBCD the virus remained infectious, although this infectivity was significantly ($p<0.05$) reduced compared to the untreated control. With further cholesterol removal, infectivity was dramatically decreased so that at concentrations of 2.5mM MBCD and above (<10% cholesterol remaining), viral infectivity was indistinguishable from the lower limit of the infectivity assay.

Assessment of fusion ability of viral samples was quantified using a syncytium induction assay, in which HIV-1\textsubscript{MN} was treated with increasing concentrations of MBCD and titrated on C8166 cells before observing syncytia formation. Table 3.1 shows that cholesterol depletion detrimentally affected viral infectivity at the fusion stage, as reduced numbers of syncytia were observed even at low (1mM) concentrations of MBCD. However, no change in fusion ability was observed using 0.5mM, reflecting the minimal loss of cholesterol seen in Figure 3.1 at this concentration.
Figure 3.1. The laboratory-adapted HIV-1 strain, HIV-1\textsubscript{MN} (10\textsuperscript{5} TCID\textsubscript{50}/ml), was incubated with increasing concentrations of MBCD (x-axis) for 1 hour at 37\textdegree C before purification (Materials and Methods, section 2.8). Envelope cholesterol content was quantified using the Amplex Red Cholesterol Kit (section 2.9). Figure 3.1 shows the percentage cholesterol (y-axis) in comparison to untreated HIV-1\textsubscript{MN}. To account for background cholesterol, viral samples were corrected by subtraction of the cholesterol quantified from uninfected cell supernatant treated with identical concentrations of MBCD. Error bars display the standard deviation calculated from a single experiment carried out in triplicate. Assays were repeated 3 times to ensure consistency of results. The \(p\) value reflecting the difference between untreated and 1mM MBCD-treated virus is shown. Statistical tests in Figures 3.1-3.5 were performed to analyse significant differences between untreated and 1mM MBCD-treated HIV-1\textsubscript{MN}. This indicated changes in viral cholesterol, infectivity and protein content at a level of cholesterol depletion important for later neutralisation and antibody binding experiments.
Figure 3.2. Effect of MBCD treatment on HIV-1 infectivity.

\[ p < 0.05 \]

The infectivity of HIV-1MN, after treatment with increasing amounts of MBCD (x-axis), was assessed in an NP2 virus titration assay (Materials and Methods, section 2.10). Viral infectivity was measured in focus forming units (FFU) and the percentage infectivity calculated against an untreated HIV-1MN control (y-axis). The \( p \) value between untreated and 1mM MBCD-treated virus is shown. Error bars display the standard deviation calculated from a single experiment carried out in triplicate. Assays were repeated 3 times to ensure consistency of results.
Table 3.1. The effect of MBCD treatment on virus syncytium induction.

<table>
<thead>
<tr>
<th>MBCD concentration (mM)</th>
<th>Syncytia</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>+ + +</td>
</tr>
<tr>
<td>0.5</td>
<td>+ + +</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>2.5</td>
<td>+</td>
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<td>3.5</td>
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<td>5</td>
<td>-</td>
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<tr>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>12.5</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3.1 shows syncytium induction by titration of MBCD-treated virus onto C8166 cells (Materials and Methods, section 2.11). The laboratory-adapted HIV-1 strain, HIV-1$_{MN}$ ($10^5$ TCID$_{50}$/ml), was incubated with increasing concentrations of MBCD (x-axis) for 1 hour at 37°C before purification (section 2.8). Purified virus was serially diluted 10-fold (from 1/10 to $10^6$) before being added to C8166 cells in triplicate. Data displayed are those observed at a 1/10 viral dilution. Syncytia production was scored and + signs represent syncytia counted, + more than 5 syncytia, + + more than 15, + + + more than 25 and - syncytia not observed.
The effect of MBCD treatment on viral RT activity.

To assess the impact of cholesterol depletion on HIV-1MN RT activity, supernatant from cells chronically infected with HIV-1MN was treated with increasing MBCD (Materials and Methods, section 2.8). After purification of virus to remove MBCD, RT activity was assessed by quantitative RT-PCR (qRT-PCR).

An overall reduction in RT activity with increasing MBCD concentration was observed (Figure 3.3), however, there was no significant change in RT activity at MBCD concentrations of up to 1mM ($p=0.513$). At MBCD concentrations equal to or greater than 2.5mM, RT activity declined and was undetectable at 10mM and 12.5mM MBCD.

The effect of MBCD treatment on viral protein composition.

The protein composition of HIV-1MN treated with increasing concentrations of MBCD was determined by quantifying two key viral proteins, p24 and gp120. Supernatant from HIV-1MN chronically infected cells was treated with increasing MBCD and p24 or gp120 content quantified by ELISA and Western Blot, after purification of intact virions (Materials and Methods, sections 2.13. – 2.15).

Viral p24 content was maintained following MBCD treatment up to a threshold MBCD concentration of 3.5mM (Figure 3.4), above this point, there was a significant reduction in p24 ($p<0.001$). This suggests that viral permeabilisation occurs at higher concentrations than 3.5mM MBCD, with subsequent loss of p24 and RT activity (Graham et al., 2003). By contrast, viral gp120 content (Figure 3.5) was unchanged across the entire range of MBCD concentrations used and this illustrates the potential for using cholesterol removal in whole-inactivated vaccine design, as the quantity of gp120 antigen remained constant.

Viral p24 and gp120 composition were confirmed by Western blot (Figure 3.6). Detection of gp120 using a commercial antibody (Figure 3.6A) resolved bands corresponding to gp120 that were unchanged for every concentration of MBCD used. The amount of p24 remained constant up to 2.5mM MBCD, but was reduced at higher concentrations in the same Western Blot. Analysis of other viral proteins probed using
Figure 3.3. HIV-1MN was treated with MBCD (x-axis), as described in the legend for Figure 3.1. The purified virus was assayed for RT activity, quantified using qRT-PCR (Materials and Methods, section 2.12) and displayed in mU/ml (y-axis). The $p$ value indicating the difference between untreated and 1mM MBCD-treated virus is shown. Error bars display the standard error calculated from three independent experiments carried out in triplicate.
Figure 3.4. Effect of MBCD treatment on HIV-1 p24 content.

Figure 3.4. HIV-1$_{MN}$ was treated with MBCD (x-axis), as described in the legend for Figure 3.1. The viral p24 content (y-axis) post-cholesterol depletion was quantified by ELISA using uninfected cell supernatant as the negative control (Materials and Methods, section 2.13). The $p$ value illustrating the difference between untreated and 1mM MBCD-treated virus is shown. Error bars display the standard error calculated from three independent experiments carried out in triplicate.
Figure 3.5. Effect of MBCD treatment on virion-bound HIV-1 gp120 content.

Figure 3.5. HIV-1_{MN} was treated with MBCD (x-axis), as described in the legend for Figure 3.1. Viral gp120 content (y-axis) was quantified by ELISA (Materials and Methods, section 2.14) using uninfected cell supernatant as the negative control. The \( p \) value illustrating the difference between untreated and 1mM MBCD-treated virus is shown. Error bars display the standard error calculated from three independent assays carried out in triplicate.
Figure 3.6 shows Western blots of the proteins from cholesterol-depleted HIV-1MN and an untreated (0mM MBCD) control. Virus was treated with MBCD (1-10mM concentrations), purified, the proteins separated using a 4-20% Tris-glycine gradient gel (Novex) and transferred onto a nitrocellulose membrane (Materials and Methods, section 2.15). **A.** gp120 was probed using rabbit polyclonal antiserum and p24 detected using a mouse monoclonal antibody (Materials and Methods, section 2.15). Positive controls (lanes 1 and 8 from the left) included recombinant HIV-1IIIB gp120 and recombinant p24. **B.** Pooled HIV-1 patient antiserum was used to visualise other viral proteins (Materials and Methods, section 2.15). The negative control (negative) consisted of uninfected cell supernatant.
pooled HIV-1 patient antiserum (Figure 3.6B) indicated no change in unprocessed p55 (Gag) and no loss of virus-associated IN with MBCD treatment. Western blots probed with pooled HIV-1 patient antiserum confirmed a reduction in RT (p51 in Figure 3.6B) with MBCD concentrations higher than 2.5mM.

The effect of MBCD treatment on HIV-1\textsubscript{MN} morphology.

To determine if MBCD affected virus morphology, purified HIV-1\textsubscript{MN} treated with a range of MBCD concentrations (0-12.5mM) was subjected to negative staining by uranyl acetate and virions observed by transmission electron microscopy (TEM) (Materials and Methods, section 2.16). Uninfected cell supernatant treated in parallel as a control had some particulate background material, however, no virus-sized particles were observed (data not shown). Images of HIV-1\textsubscript{MN} (Figure 3.7A-E) show that, despite increasing concentrations of MBCD, virus particles remain intact and virus morphology was unchanged at MBCD concentrations up to 5mM compared to untreated virus (Figure 3.7A-C). At concentrations greater than or equal to 5mM (Figure 3.7D and E), virions became more hexagonal in shape. The virus also appeared to be more densely stained upon cholesterol removal, suggesting permeabilisation of the virus envelope and penetration of the negative stain into the virus structure.

Changes in the interaction of HIV-1\textsubscript{MN} with MAbs after cholesterol depletion.

Indirect antibody binding assays were developed to quantitate MAb binding post-cholesterol depletion. These assays consisted of capturing intact virus on ELISA plates before quantification of bound virus by p24 content or RT activity. Estimation of direct MAb binding by sandwich ELISA was attempted, but due to high non-specific binding of the secondary antibodies tested, indirect assays were developed as an alternative. Using these indirect assays, the binding of three MAbs, 2F5, b12 and 2G12 directed to the MPER gp41 region, the CD4 binding site and viral carbohydrate residues, respectively, were assessed.

By modification of a published HIV-1 assay (Nyambi \textit{et al.}, 2001), adsorption of purified virions to ELISA plates pre-coated with each MAb was quantified by assaying bound virus by p24 (Materials and Methods, section 2.18). Figure 3.8 shows the
Figure 3.7. Transmission electron microscopy of MBCD-treated HIV-1.

Figure 3.7. HIV-1_{MN} was treated with MBCD (Materials and Methods, section 2.8) and purified by ultracentrifugation through a 20% sucrose cushion. The virus was resuspended in PBS containing 0.5% gluteraldehyde, negatively stained and observed by transmission electron microscopy (section 2.16) at 42,000 times magnification. The following concentrations of MBCD were used in Figures A-E: A – Untreated HIV-1_{MN}, B – 1mM MBCD, C – 2.5mM MBCD, D – 5mM MBCD, E – 10mM MBCD, F – 0mM MBCD.
Figure 3.8. Changes in MAb immunoprecipitation of HIV-1 post-cholesterol depletion.

Figure 3.8. MAbs (2F5, b12 and 2G12) were incubated with cholesterol-depleted HIV-1\textsubscript{MN} (dotted bars, treated with 1mM MBCD) and the capture of virus compared to the untreated viral control (solid bars). Virus was bound to immobilised MAb and lysed before quantification of bound virus by p24 ELISA (Materials and Methods, section 2.13). A negative control consisting of uninfected cell supernatant was treated in parallel, with or without 1mM MBCD. Bars represent absorbance measured at 492nm, corrected for background absorbance derived from the negative controls. Error bars indicate the standard deviation of the mean from a single experiment.
capture of cholesterol-depleted HIV-1\textsubscript{MN} by MAbs, compared to untreated virus. An increase in bound virus was recorded with the MAbs 2F5 and b12, but statistically this was not significant. This was due to low differences between the cut-off value and sample absorbance readings, limiting the resolution of changes in MAb binding after cholesterol depletion. In addition to this, capture of virus to 2G12 could not be distinguished from background levels, most likely due to the low affinity of this MAb for HIV-1\textsubscript{MN} (maximum of 25% neutralisation at 2µg/ml in Figure 3.13).

Therefore, the interaction of MAbs with HIV-1\textsubscript{MN} was assayed by quantifying bound virus using RT activity in order to measure indirectly significant differences in antibody binding. A sensitive qRT-PCR method, developed in the laboratory (Pizzato et al., 2008) illustrated a 4-fold difference in 2F5 binding compared to native virus (Figure 3.9).

As quantifying bound virus by RT activity was more successful at measuring differences in MAb binding to cholesterol-depleted virus, additional MAbs were tested. Two-fold increases in bound virus with 4E10 and 4.8D were recorded post-cholesterol removal. The MAb 4.8D is a CD4i antibody that binds to the co-receptor binding site of gp160 (section 1.9.3). This increased association of 4.8D with HIV-1\textsubscript{MN} illustrates that exposure of the virus co-receptor binding site occurs after cholesterol depletion without the need for prior sCD4 induction. There was no increase in b12 binding, however, most likely due to the already high affinity of this antibody. Binding of virus by the 2G12 MAb was unaffected by cholesterol depletion. This is in contrast to the findings of the neutralisation assay (Figure 3.13) where up to 15% increases in viral neutralisation after cholesterol depletion were observed.

To illustrate the specificity of the virus capture assay, non-specific viral adsorption to MAbs was tested with samples consisting of virus incubated with competing recombinant gp120. Similarly, to determine any non-specific binding to viral proteins other than gp120, HIV-1\textsubscript{NL4-3} containing no envelope glycoprotein was used. In these cases, the RT activity was equal to that of uninfected cell supernatant (data not shown).
Figure 3.9. Changes in MAb immunoprecipitation of cholesterol-depleted HIV-1.

![Graph showing fold increase in MAb immunoprecipitation of cholesterol-depleted HIV-1 MN](image)

**Figure 3.9.** MAb immunoprecipitation of cholesterol-depleted HIV-1\textsubscript{MN} illustrated as fold increase compared to native virus. MAb bound virus was quantified by RT activity (Materials and Methods, section 2.12). The figure displays cumulative data, from three separate experiments, of virus binding to the MAb’s b12, 2F5, 2G12, 4E10 and 4.8D. Antibody binding to cholesterol-depleted virus is shown as -fold increase compared to the untreated virus control. Error bars display the standard deviation of the mean of triplicate samples from three independent experiments.
The effect of cholesterol depletion on HIV-1MN neutralisation

To assess the impact of cholesterol depletion on glycoprotein antigenicity, changes in HIV-1MN neutralisation with 1mM MBCD treatment was compared to an untreated control. Since infectious virus is needed to assess neutralisation by cell-based assays, 1mM MBCD treatment was chosen. At this concentration, MBCD significantly ($p<0.05$) reduced envelope cholesterol content, by ~50%, while maintaining 30% viral infectivity when compared to untreated virus (Figures 3.1 and 3.2). Cholesterol depletion of HIV-1MN by 1mM MBCD had no effect on viral protein composition or morphology (Figures 3.3-3.5).

A panel of neutralising MAbs directed to different epitopes within trimeric gp160 was exploited to investigate the effect of cholesterol removal from the virus envelope on neutralisation. Neutralisation was assessed using the MAbs, b12, 447-52D, 2F5, 4E10, 2G12, along with sCD4 and pooled HIV-1 patient antiserum (Clade B). Differences in neutralisation compared to the untreated control could indicate a change in trimeric glycoprotein conformation and antigenicity as a result of cholesterol depletion.

The b12 antibody is a potent neutralising MAb which targets the CD4 binding site (Barbas et al., 1992; Roben et al., 1994). Following HIV-1 cholesterol depletion, neutralisation by b12 (Figure 3.10) was significantly ($p<0.05$) increased by 40% or more at MAb concentrations below 0.5µg/ml. Above this concentration, inhibition of infectivity reached a plateau (>80% viral inhibition) and further differences in neutralisation after cholesterol depletion could not be resolved. Importantly, cholesterol depletion of HIV-1MN facilitated achievement of peak neutralisation at a lower concentration (0.1µg/ml) than the untreated virus, reaching saturated neutralisation at 0.5µg/ml.

To explore the changes in neutralisation at an exposed epitope in the V3 region, cholesterol-depleted virus was analysed using the neutralising antibody 447-52D (Conley et al., 1994). Changes in the HIV-1MN neutralisation profile were only seen at 0.01µg/ml where cholesterol depletion significantly ($p=0.05$) reduced neutralisation by 20% (Figure 3.11). At all other concentrations 100% neutralisation was reached for both native and cholesterol-depleted virus.
Figure 3.10. Effect of cholesterol depletion on HIV-1$_{MN}$ neutralisation by a MAb targeting the CD4 binding site.

**Figure 3.10.** HIV-1$_{MN}$ was treated with 1mM MBCD (dotted line) for 1 hour at 37ºC (Materials and Methods, section 2.8) and purified by ultracentrifugation through a 20% sucrose cushion. The MAb b12 was pre-incubated in increasing concentrations (x-axis) with virus for 1 hour at 37ºC and virus neutralisation assayed by NP2 cells (Materials and Methods, section 2.17). Untreated HIV-1$_{MN}$ was included as the control (solid line). In all experiments, percentage inhibition of infectivity (y-axis) was calculated using samples incubated without MAb. Error bars display the standard deviation calculated from three independent experiments carried out in triplicate. Relevant $p$ values indicating differences between 1mM MBCD-treated virus and the untreated control are indicated with arrows, $^* = p < 0.05$. 
Figure 3.11. Effect of cholesterol depletion on HIV-1<sub>MN</sub> neutralisation by a MAb targeting the V3 region of gp120.

**Figure 3.11.** HIV-1<sub>MN</sub> was treated with 1mM MBCD (dotted line) and purified as described in the legend for Figure 3.8. The effect of the MAb, 447-52D, on virus infectivity was assessed using the NP2 neutralisation assay (Materials and Methods, section 2.17). The human MAb, 447-52D, in increasing concentrations (x-axis) was pre-incubated with virus for 1 hour at 37°C. Untreated HIV-1<sub>MN</sub> was the control (solid line). Error bars display the standard deviation calculated from two independent experiments carried out in triplicate. Relevant p values indicating differences between 1mM MBCD-treated virus and the untreated control are indicated with arrows, * = p< 0.05.
Two broadly neutralising MAbs, 2F5 and 4E10 (Muster et al., 1993), raised against the conserved MPER region of gp41 were employed to explore the exposure of these epitopes post-cholesterol depletion. These MAbs only partially neutralise HIV-1MN infectivity, but the degree of neutralisation was enhanced when cholesterol was removed. Neutralisation of HIV-1MN by the MAb 2F5 was minimal up to a threshold concentration of 0.5µg/ml (Figure 3.12A). However, at concentrations of 1µg/ml and above, cholesterol depletion increased viral neutralisation by over 20%.

Virus neutralisation by 4E10 was also increased after cholesterol depletion by 10-25% at antibody concentrations of 0.5µg/ml and 2µg/ml, respectively (Figure 3.12B). With 1µg/ml of 4E10, no difference in viral neutralisation with cholesterol depletion was observed. Therefore, an increase in 2F5 and 4E10 neutralisation after removal of cholesterol suggests some exposure of binding epitopes which are in close proximity to the viral envelope.

The conformation of carbohydrate moieties in the V3 and V4 variable regions were assessed post-cholesterol removal using the neutralising MAb, 2G12. Neutralisation of HIV-1MN by 2G12 (Figure 3.13) was increased, by 5-15% at all concentrations tested after viral envelope cholesterol depletion, but to a lesser extent than that seen with 2F5 and 4E10. Changes in the lipid membrane environment that causes small alterations to integral protein conformation, increasing the exposure of the 2G12 binding site may underpin the effect that cholesterol depletion has on epitopes located further from the membrane, such as the glycosylated 2G12 epitope. However, further data will be required to confirm this hypothesis as antibody binding and neutralisation data for 2G12 was not conclusive.

The ability of sCD4 to bind to the HIV-1MN gp120 CD4 binding site after cholesterol depletion was investigated. The hypothesis that there are subtle changes in the gp160 conformation following cholesterol depletion was confirmed by the observed increases, of between 10% and 40%, in sCD4 neutralisation of cholesterol-depleted HIV-1MN compared to untreated controls (Figure 3.14). The CD4 binding site is masked in the native gp160 trimer of many HIV-1 isolates, although less so on laboratory strains compared to primary isolates (Sattentau et al., 1993) and, therefore, slight changes in protein conformation as a result of cholesterol depletion may expose this area.
Figure 3.12. Neutralisation of cholesterol-depleted HIV-1 by MAbs targeting gp41.

**Figure 3.12.** HIV-1\textsubscript{MN} was treated with MBCD (dotted line), purified as described in the legend for Figure 3.8 and virus neutralisation determined (Materials and Methods, section 2.17). Untreated HIV-1\textsubscript{MN} was the control (solid line). The MAbs 2F5 (A) and 4E10 (B), in a range of concentrations (x-axis), were pre-incubated with virus for 1 hour at 37ºC. Error bars display the standard deviation calculated from two independent experiments carried out in triplicate.
Figure 3.13. Effect of cholesterol depletion on HIV-1 neutralisation by a MAb targeting glycosylated residues of the V3 and V4 variable regions of gp120.

**Figure 3.13.** HIV-1\textsubscript{MN} was treated with MBCD (dotted line) and purified as described in the legend for Figure 3.8. The virus was incubated with the MAb (x-axis), 2G12, for 1 hour at 37°C and neutralisation assayed by the NP2 assay. Inhibition of infectivity is expressed as a percentage of virus incubated without MAb (y-axis) (Materials and Methods, section 2.17). Untreated HIV-1\textsubscript{MN} was the control (solid line). Error bars display the standard error calculated from two independent experiments carried out in triplicate. The $p$ values indicating significant differences between 1mM MBCD-treated virus and untreated controls are indicated, * = $p< 0.05$. 
Figure 3.14. Effect of cholesterol depletion on HIV-1 neutralisation by sCD4.

Figure 3.14. HIV-1MN was treated with 1mM MBCD (dotted line) for 1 hour at 37°C (Materials and Methods, section 2.8) and purified by ultracentrifugation through a 20% sucrose cushion. Neutralisation of the virus by sCD4 was then assayed by NP2 neutralisation assay (Materials and Methods, section 2.17). The control experiment using untreated HIV-1MN is shown as a solid line. Error bars display the standard error calculated from two independent experiments carried out in triplicate. Relevant $p$ values between 1mM MBCD-treated virus and untreated controls are indicated, $* = p < 0.05$. 

...
Finally, pooled HIV-1 patient antiserum was used in neutralisation experiments to determine the effect of cholesterol removal on neutralisation by antibodies raised \textit{in vivo}. Inhibition of virus infectivity using pooled patient sera (B-clade) was greater following cholesterol depletion of HIV-1\textsubscript{MN}, compared to untreated virus (Figure 3.15). Increases in neutralisation by up to 30\% (at 1/500 and 1/4000 dilutions of antisera) were observed. The amount of neutralising serum used in these experiments was finite and in order to obtain consistent results using the same source of serum, it was not possible to study changes in neutralisation at serum dilutions of less than 1 in 500.

Table 3.2 summarises the changes in antibody neutralisation after cholesterol depletion. Inhibitory concentrations (IC) effecting 30\% and 50\% neutralisation are shown and indicate the changes in neutralisation after cholesterol removal, as lower concentrations of MAb are required to exhibit IC\textsubscript{30} and IC\textsubscript{50}. This table illustrates that cholesterol depletion can effect dramatic differences in IC exhibited by a 13-fold decrease in the IC\textsubscript{50} and IC\textsubscript{30} concentrations for b12 and 4- or 3-fold decreases in IC\textsubscript{50} and IC\textsubscript{30} values for the MAb 447-52D, respectively. All other MAb’s (2F5, 4E10, 2G12), sCD4 and pooled patient antisera exhibited at least a 2-fold decrease in IC\textsubscript{50} and IC\textsubscript{30} with cholesterol-depleted virus compared to untreated HIV-1\textsubscript{MN}. 
**Figure 3.15.** Effect of cholesterol depletion on HIV-1 neutralisation by pooled HIV-1 patient antiserum.

Figure 3.15. HIV-1\textsubscript{MN} was treated with 1mM MBCD (dotted line) for 1 hour at 37°C (Materials and Methods, section 2.8) and purified by ultracentrifugation through a 20% sucrose cushion. Neutralisation of the virus by pooled HIV-1 patient antiserum, serially diluted 2-fold from 1/500 (x-axis), was assayed by NP2 neutralisation assay (Materials and Methods, section 2.17). Untreated HIV-1\textsubscript{MN} was the control and is shown by a solid line. Percentage inhibition of infectivity (y-axis) was calculated using virus incubated without antisera. Error bars display the standard error calculated from two independent experiments carried out in triplicate. Relevant p values between 1mM MBCD-treated virus and untreated controls are indicated, * = p< 0.05.
Table 3.2. Effect of cholesterol depletion on MAb, sCD4 or antisera inhibitory concentrations.

<table>
<thead>
<tr>
<th>MAb/sCD4/sera</th>
<th>IC\textsubscript{50} (µg/ml)</th>
<th>IC\textsubscript{30} (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0mM MBCD</td>
<td>1mM MBCD</td>
</tr>
<tr>
<td>b12</td>
<td>0.08</td>
<td>0.006</td>
</tr>
<tr>
<td>447-52D</td>
<td>0.04</td>
<td>0.009</td>
</tr>
<tr>
<td>2F5</td>
<td>1.9</td>
<td>0.85</td>
</tr>
<tr>
<td>4E10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2G12</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>sCD4</td>
<td>0.7</td>
<td>0.3</td>
</tr>
<tr>
<td>Antisera</td>
<td>1/4000</td>
<td>1/1200</td>
</tr>
</tbody>
</table>

Table 3.2. Calculated IC\textsubscript{50} and IC\textsubscript{30} values (µg/ml) for cholesterol depleted HIV-1\textsubscript{MN} (1mM MBCD) compared to native virus (0mM MBCD). Inhibitory concentrations values were calculated from data plots displayed in Figures 3.10 to 3.15. Dashes display inhibitory concentrations for which there was no data.
3.3 Discussion

HIV-1 buds selectively from lipid rafts within the cell membrane (Aloia et al., 1993). Hence, the virus envelope is mostly lipid raft-like in composition, with an enriched cholesterol content (Nguyen et al., 2000). Although the role of lipids in the binding of MPER MAbs, like 4E10 and 2F5, has been explored (Haynes et al., 2005; Alam et al., 2007), the effect of cholesterol on the antibody binding dynamics of gp160 at a wide range of epitopes of whole virus particles, has never been investigated. In this chapter, the effect of cholesterol depletion on viral infectivity, composition, antibody binding and neutralisation of HIV-1MN was explored.

The laboratory-adapted HIV-1 strain, MN, was incubated with increasing concentrations of MBCD and the resulting removal of cholesterol followed a biphasic pattern (Figure 3.1). These observations correlate with previously published data using an unmethylated homolog of MBCD, 2-hydroxy-propyl-β-cyclodextrin, to deplete cholesterol from both SIVMNE and HIV-1MN (Graham et al., 2003). This biphasic pattern previously reported (Graham et al., 2003) and typified by slow, initial cholesterol removal followed by rapid decreases to minimal levels, indicates lipid-raft pools rich in cholesterol within the viral envelope. Cholesterol tightly bound in rafts is not removed until a threshold concentration of MBCD is met, whereas cholesterol not associated with lipid rafts is efficiently removed (Graham et al., 2003).

The relationship between cholesterol depletion and viral infectivity is dose-dependent, as increasing MBCD concentrations reduced infectivity to below detectable levels. This reduction was not due to changes in protein composition, as both RT activity and p24 remained constant at concentrations of MBCD associated with a loss of infectivity. Both viral RT activity and p24 content declined above an MBCD threshold (of 2.5mM and 3.5mM MBCD, respectively), representing viral envelope permeabilisation at the stage where envelope cholesterol is almost entirely removed and, thus, internal proteins are lost (Graham et al., 2003). The reason for RT loss from cholesterol-depleted virus at lower concentrations than observed for p24 is unclear. Recombinant RT was incubated with MBCD to determine if losses in RT activity were due to MBCD directly, however, no reduction of recombinant RT activity was observed up to 12.5mM concentrations (data not shown).
Despite a loss of internal proteins at maximal cholesterol depletion, envelope glycoprotein levels remained constant throughout, as was shown in Figure 3.5 and 3.6. This would be important if cholesterol-depleted whole virus was to be considered as a vaccine immunogen because glycoprotein is the key immunogen needed to induce neutralising antibodies post-immunisation.

Reduction in viral infectivity after cholesterol depletion results from inhibition of viral fusion post-receptor binding (Manes et al., 2000; Liao et al., 2001; Guyader et al., 2002; Ablan et al., 2006). Virus permeabilisation and the loss of internal viral proteins using higher concentrations of MBCD may contribute to reducing viral infectivity. Graham et al. (2003) suggested that infectivity is not totally ablated until the tightly bound cholesterol pool associated with lipid rafts is removed, corresponding to the MBCD concentration threshold at which virus permeabilisation occurs. This reduction in infectivity is specific to cholesterol depletion, as cholesterol replenishment can restore HIV-1 infectivity (Manes et al., 2000; Campbell et al., 2002), however, replacing cholesterol with homologs, such as cholestenone or sphingolmyelin, does not restore infectivity (Campbell et al., 2002).

Two groups have been able to replace cholesterol after depletion and restore inhibition of viral fusion (Manes et al., 2000; Campbell et al., 2002). This replenishment of cholesterol into the viral envelope after cholesterol removal has been done using cholesterol-chelated MBCD or exogenous cholesterol (Manes et al., 2000; Campbell et al., 2002). Although attempts were made in the present study to investigate cholesterol replenishment, further work would be needed to establish conditions whereby the infectivity of the virus could be retained as a result. On purification of the virus after addition of cholesterol-chelated MBCD a white residue was observed at the top of the sucrose gradient. This could have been surplus lipid, however, it is possible that the addition of cholesterol back into the virus resulted in destruction of the particles leading to the loss of infectivity observed. Therefore, despite repeated attempts optimisation of the correct concentration of cholesterol-chelated MBCD needed to maintain viral infectivity whilst replenishing the cholesterol removed would be needed.
Negative staining of HIV-1MN, from which cholesterol had been removed from the envelope, showed that virions remained intact. Penetration of the negative stain into the virus was indicated by the dark appearance of the particles compared to the untreated control. This was seen even at low (1mM) concentrations of MBCD. Above the point of viral permeabilisation (2.5mM MBCD treatment), virions appeared hexagonal in shape. This may be due to the collapse of particles, resulting from the loss of internal structures formed by the capsid and matrix proteins. Negative staining did not produce sharp images with fine resolution of internal viral structures or glycoprotein spikes, however, it did allow the integrity of HIV-1 particles to be assessed.

Images taken by Graham et al. (2003), using viral sectioning techniques, clearly shows maintenance of viral morphology with cholesterol depletion, but before permeabilisation when small holes appear in the virus envelope. The TEM images in this 2003 study, illustrate that β-cyclodextrin compounds may pluck discrete areas of cholesterol from the viral membrane. Therefore, investigation of milder cholesterol modifying processes, such as di-isopropyl ether (DIPE) treatment (Kitabwalla et al., 2005), may be useful in maintaining internal viral proteins at higher levels of envelope cholesterol removal.

A MAb immunoprecipitation assay was developed to capture virus and indirectly assess the antibody binding of HIV-1MN after cholesterol depletion. This assay utilised quantitative RT-PCR (Pizzato et al., 2008) to estimate MAb capture of HIV-1MN and was more sensitive than quantifying virus by p24 ELISA. This ELISA required a delicate balance of the concentration of antibody and the amount of virus, so as not to either saturate the antibody or bind all virions present in the sample. Due to the affinity of HIV-1MN binding by the MAb b12, no increase in b12 immunoprecipitation of virus was observed after cholesterol removal. It may have been possible to resolve a difference in b12 MAb immunoprecipitation of HIV-1 with cholesterol depletion by titrating virus to determine the correct concentration at which the b12 is not saturated on the ELISA plate.

The effect of reducing the cholesterol content on virus neutralisation and antibody binding was investigated to ascertain the effect of high levels of cholesterol in the viral envelope on glycoprotein antigenicity (Aloia et al., 1988; Aloia et al., 1993). Increases
in neutralisation using MAbs that have potent neutralising ability against HIV-1MN, for example b12 and 447-52D, were difficult to resolve. This may be due to the efficient neutralisation and high affinity of these MAbs for HIV-1MN. However, the concentration of b12 at optimal neutralisation was lower for cholesterol-depleted virus compared to the untreated control.

For those MAb’s that effected partial viral neutralisation, such as 2F5, 4E10 and 2G12, increases in antibody binding and infectivity inhibition after cholesterol depletion were more easily analysed. Neutralisation and antibody binding was increased post-cholesterol depletion for 2F5 and 4E10, compared to an untreated control. Despite 10% increases in 2G12 viral neutralisation with cholesterol removal, a similar increase in 2G12 binding could not be recorded due to the low affinity of this antibody for HIV-1MN (maximum neutralisation of 25% with native virus). The ability of sCD4 to neutralise virus after cholesterol depletion was enhanced by 10-40% for all the concentrations of sCD4 tested.

Data derived from these experiments suggests exposure of viral epitopes, due to subtle conformational changes allowing increased antibody binding and neutralisation at binding sites in gp41 (for example, 2F5 and 4E10, which bind at the MPER region of gp41). Increased neutralisation of HIV-1 with cholesterol depletion, by 2G12 and sCD4, could be accounted for by exposure of epitopes distant from the viral envelope. An increase in viral neutralisation by pooled HIV-1 patient antiserum would further suggest exposure of diverse viral epitopes following cholesterol removal. Therefore, reductions in envelope cholesterol may allow increased flexibility of the HIV-1 glycoproteins within a more fluid membrane that could expose glycoprotein epitopes, like 2F5 or 4E10, which are tightly associated with the viral envelope (Haynes et al., 2005; Alam et al., 2007; Sun et al., 2008). Compounded with this, subtle glycoprotein conformational changes as a result of depletion of cholesterol from the virus lipid membrane may lead to the exposure of gp120 epitopes affecting sites distant from the virus envelope.

The greatest changes in MAb immunoprecipitation and neutralisation with cholesterol removal from virus occurred with the MAbs 2F5 and 4E10. Due to the unique nature of MPER epitopes, whereby several amino acids are buried within the viral envelope (Sun
et al., 2008), cholesterol depletion may uncover these residues from the lipid environment to enhance MAb binding. An induced-fit model has been described for the binding of 4E10 and 2F5, in which lipid contacts initiate the association of the MAb with HIV-1, followed by the unmasking of lipid-buried residues and high affinity binding resulting in viral neutralisation (Haynes et al., 2005; Alam et al., 2007; Sun et al., 2008).

The Mab 4E10 directed against the MPER region of gp41 has been shown to bind to phospholipids, cholesterol and cardiolipin, in particular (Haynes et al., 2005; Alam et al., 2007; Matyas et al., 2009). These lipid interactions are thought to be important in antibody binding to membrane-inserted epitopes allowing neutralisation of virus by interfering with membrane fusion (Huarte et al., 2008). Although 2F5 also binds in this region of gp41 and has been shown to bind to glycolipids and ceramide, its lipid binding properties are more restricted and do not include cardiolipin or cholesterol (Haynes et al., 2005; Alam et al., 2007; Matyas et al., 2009). Hence, removal of cholesterol could enrich the proportion of these lipids at the viral surface and encourage MAb binding to the viral membrane (Alam et al., 2007). To test this theory, the binding of anti-cardiolipin autoantibodies (Haynes et al., 2005) to cholesterol-depleted HIV-1MN, compared to an untreated control, may reveal the lipid binding dynamics to virus. Competition experiments with 4E10 and anti-cardiolipin autoantibodies could also reveal whether the changes in neutralisation observed were due to differences in MAb lipid binding, direct epitope binding or both.

Huate et al. (2008) utilised an HIV-1 gp41 peptide (amino acids 656-683 encompassing the full 4E10 and 2F5 epitopes) inserted into lipid vesicles of different lipid compositions to explore the binding of the 4E10 and 2F5 MAb’s (Huarte et al., 2008). The lipid vesicles used were made of palmitoyl-2-oleylphosphatidylcholine (POPC) with or without cholesterol or sphingomyelin (POPC:CHOL and POPC:SPM, respectively) and encapsulated fluorometric substrates (Huarte et al., 2008). Peptide insertion into the vesicles induced leakage of the fluorometric substrate, which was inhibited by the binding of the 4E10 or 2F5 antibodies. Lower concentrations of 4E10 were required to arrest leakage in POPC and POPC:CHOL vesicles compared to 2F5 (Huarte et al., 2008).
The addition of cholesterol into the POPC vesicles induced a shallower insertion of the peptide into the membrane, without affecting secondary peptide structure and led to a higher inhibitory capacity of vesicle leakage by the antibody 4E10 (Huarte et al., 2008). However, the inhibition of vesicle leakage on the addition of sphingomyelin (POPC:SM) was reduced with both 4E10 and 2F5 in comparison with POPC and POPC:CHOL vesicles (Huarte et al., 2008). These results show that membrane composition can affect the binding dynamics of the 4E10 and 2F5 antibodies and that the lipid environment impacts on epitope accessibility (Huarte et al., 2008).

The POPC:CHOL vesicles used in the Huarte et al. (2008) study contained molar ratios of cholesterol:POPC of 2:1. In the present study we have demonstrated greater accessibility of the 4E10 and 2F5 epitopes in HIV-1 envelope environments with reduced cholesterol. It has been shown that the cholesterol:PC molar ratio of HIV-1NL4-3 is 5:1 (Brugger et al., 2006) which is much higher than the concentrations used by Huarte et al. (2008). Therefore, it would be important to extend these vesicle experiments to utilise higher cholesterol:PC ratios in order to explore the accessibility of epitopes within membranes mimicking the wildtype virus envelope and then compare these to vesicles with lower cholesterol concentrations.

Due to the requirement of infectious virus in cell-based neutralisation assays, a concentration of MBCD that retained some HIV-1MN infectivity (1mM MBCD) had to be chosen. Analysis of cholesterol-depletion of HIV-1MN with MBCD concentrations in excess of 1mM could be done using the antibody binding ELISA developed. This could identify the concentration of MBCD creating the optimum glycoprotein antigen exposure post-cholesterol removal. It would also be of interest to investigate the regions of gp120 exposed following cholesterol depletion using a larger panel of MAbs raised against other glycoprotein regions and epitopes (Moore et al., 1994). Probing the structure of cholesterol-depleted HIV-1MN using MAbs targeting all conserved or variable domains could lead to a clearer picture of glycoprotein epitope exposure with cholesterol depletion (Moore et al., 1993; Moore et al., 1993; Moore et al., 1994).

To support the hypothesis of viral epitope exposure on cholesterol depletion, unpublished data from the laboratory involving the viral accessory protein Nef also points to the role of cholesterol in glycoprotein conformation (Lai, 2009). The Nef
protein promotes cholesterol production (van 't Wout et al., 2005) and inhibits cholesterol efflux (Mujawar et al., 2006) in an infected cell, elevating cell membrane cholesterol and, hence, HIV-1 envelope cholesterol upon budding. Therefore, Nef-deficient virus has been documented to contain decreased amounts of cholesterol in the viral envelope (van ’t Wout et al., 2005). Data comparing Nef-deficient HIV-1_{NL4-3} with Nef-positive virus resulted in significant increases in viral neutralisation and antibody binding using the MAb’s 2F5, b12 and 2G12 in the absence of Nef (Lai, 2009).

We have shown that cholesterol depletion can increase the neutralisation of and antibody binding to HIV-1_{MN}. We hypothesise that this is due to changes in the fluidity of the viral envelope that impacts on the flexibility of gp160, exposing masked epitopes close to the membrane. Compounded with this, cholesterol depletion could cause slight changes in protein conformation that result in increases in antibody binding to envelope glycoproteins and viral neutralisation. These discoveries may have important applications in the vaccine field where modification of immunogens to elicit broad antibody responses to HIV-1 is an important target.
Chapter 4:

Cholesterol depletion of an HIV-1 pseudotype and its effect on Env antigenicity.
4.1 Introduction

During virus culture, gp120 is readily released into the culture medium, however, the amount of gp120 release differs depending on the HIV-1 isolate in question (Schneider et al., 1986; McKeating et al., 1991). McKeating et al. (1991) have shown that after 48 hours of co-culturing infected H9 cells with uninfected cells, HIV-1RF and HIV-1IIIB (originally named HTLV-IIIIB) (Popovic et al., 1984) released less gp120 into the culture medium compared to HIV-1SF-2 (originally named ARV-2 (Levy et al., 1984)). These three isolates showed losses of gp120 into the culture medium in quantities of 1.2 ng, 0.7 ng and 3.5 ng, respectively.

The HIV-1 Gag:Env ratios for the HIV-1 isolates RF, IIIB and SF-2 are 14:1, 17:1 and 64:1, respectively (McKeating et al., 1991). It has also been shown that the amount of virion bound gp120 on HIV-1IIIB (4.8 ng) and HIV-1RF (4 ng) is higher than that on HIV-1SF-2 (0.57 ng). These data indicate that the amount of gp120 released during culture and the Gag:Env ratio vary with different viral isolates and that these factors can indicate the amount of virion-bound gp120 for different HIV-1 isolates.

It has been described that laboratory-adapted isolates of HIV-1 spontaneously shed gp120 over time during in vitro culture at 37°C and that this loss in gp120 coincides with a reduction in viral infectivity (McKeating et al., 1991; Layne et al., 1992; Moore et al., 1992; Hammonds et al., 2003). This is partly due to the weak non-covalent interactions between gp120 and gp41 (Schneider et al., 1986; McKeating et al., 1991; Moore et al., 1992) and, hence, gp120 is readily released into the culture supernatant during viral aging, purification processes or experimental manipulation (Schneider et al., 1986; McKeating et al., 1991).

Investigations by McKeating et al. (1991) indicated that, despite possessing low amounts of virion-bound gp120, the HIV-1 strain, SF-2, possesses stable gp120:gp41 trimers, reducing the amount of glycoprotein shed (only 20% gp120 is shed over 24 hours at 37°C). This is in contrast to HIV-1IIIB, HIV-1RF and HIV-1MN, which exhibit glycoprotein shedding over time with a subsequent loss in infectivity (McKeating et al., 1991; Moore et al., 1992; Hammonds et al., 2003). In the case of HIV-1RF, 75% of its virion-bound gp120 is lost over 24 hours of culture at 37°C (McKeating et al., 1991).
Despite these early papers suggesting that gp120 shedding is common among some HIV-1 isolates, more recently published data contradicts this idea. Moore et al. (1992) exhibited significant gp120 shedding as result of sCD4 binding to virus, however, in the absence of sCD4, gp120 shedding from HIV-1RF was minimal. In a number of other papers gp120 shedding has been shown to be minimal for a large array of both laboratory-adapted and primary isolates (for example, HIV-1 laboratory-adapted isolates, RF, NL4-3, HIV-2 isolates, LAV-2ROD and CBL20 and the simian immunodeficiency virus, SIVMAC-251) after 2-8 hours of in vitro incubation at both 4°C and 37°C (Levy et al., 1984; Moore et al., 1992; Sattentau et al., 1993; Karlsson et al., 1996; Chertova et al., 2002; Hammonds et al., 2003). In these cases, the only incidence of gp120 shedding was at temperatures exceeding 50°C (Moore et al., 1992; Chertova et al., 2002; Hammonds et al., 2003).

Moreover, these isolates did not release gp120 upon freeze-thawing or after heat treatment indicating that gp120 shedding from primary isolates is not common in vitro (McKeating et al., 1991; Moore et al., 1992; Chertova et al., 2002). The observation that cultured HIV-1 primary isolates contain relatively stable glycoprotein trimers compared to HIV-1 laboratory-adapted strains has also fuelled debate as to whether gp120 shedding is important during natural infection (Groenink et al., 1995; Chertova et al., 2002; Hammonds et al., 2003).

Therefore, the existence of gp120 shedding has been questioned, citing differences in viral gp120 incorporation rather than gp120 shedding as being important (Chertova et al., 2002). To test this, molar ratios of gp120 compared to gp41 were calculated to indicate the true extent of gp120 shedding (Chertova et al., 2002). This is an alternative method of measuring gp120 shedding that relies on the fact that non-associated glycoprotein is not incorporated into the virion due to polyprotein expression of Env (Crowl et al., 1985) and, hence, molar ratios should be 1:1 if no gp120 shedding occurs. Chertova et al. (2002) quantified the ratio of gp41 to gp120 of SIVMNE, HIV-1NL4-3 and HIV-1MN and found equimolar amounts in all strains tested.

The aim of this chapter was to identify primary isolates of HIV-1 with significantly high levels of virion-associated gp120 compared to laboratory-adapted strains. This was done with the aim of investigating the effect of cholesterol depletion on the
neutralisation profile of a primary HIV-1 isolate. A panel of laboratory-adapted and primary HIV-1 isolates were analysed for virion-bound gp120 content.

The effect of cholesterol depletion on \textit{in vitro} viral neutralisation was assessed using primary B-clade pseudotyped HIV-1 due to difficulties cultivating a primary isolate to high titre. Although using a clade C virus would have been more appropriate given that C clade viruses cause the majority of infections worldwide, it was decided to use a clade B virus as proof-of principle. This was because clade B HIV-1 is better characterised than clade C viruses. Moreover, we did not have any clade C pseudotypes at the start of this project due to the challenges involved in growing them \textit{in vitro}. 
4.2 Results

**Quantification of virion-bound gp120 in laboratory-adapted and primary HIV-1.**

The CXCR4-restricted HIV-1 laboratory-adapted isolates, MN and NL4-3, were grown for 14 days in H9 cells or in PBMCs from HIV-1 negative donors (Materials and Methods, section 2.6). The CCR5-tropic laboratory strain, JRCSF, and all primary isolates studied were cultured in HIV-1 negative PBMCs for a similar length of time. Infected cell supernatant was harvested and intact virions purified by ultracentrifugation through a 20% sucrose cushion to fractionate virions and remove soluble gp120. Samples were normalised for RT activity and viral gp120 content was quantified by ELISA (Materials and Methods, section 2.14).

The laboratory-adapted strain, NL4-3 (Figure 4.1A), contained the most gp120 (12ng/µU of RT activity). When HIV-1\textsubscript{MN} was grown in H9 cells, the amount of virion-bound gp120 was half that observed for HIV-1\textsubscript{NL4-3} (5.5ng/µU). Both laboratory-adapted strains (NL4-3 and MN), when propagated in primary cells, had more than a 2-fold reduction in gp120 content ($p<0.05$), compared to virus harvested from H9 cells. Virion-bound gp120 quantified from NL4-3 (Figure 4.1A) and grown in PBMCs was significantly higher ($p<0.05$) than the MN strain propagated in a similar way.

Figure 4.1A shows that all primary isolates incorporate less gp120 into the viral envelope than the HIV-1 laboratory-adapted, CXCR4-using strains, grown in both PBMCs and H9 cells ($p<0.001$). The CCR5-restricted laboratory-adapted strain, JRCSF, had extremely low amounts of gp120 (0.13ng/µU of RT activity, Figure 4.1A), less than most of the primary isolates tested.

Figure 4.1B shows an expanded y-axis view of Figure 4.1A and illustrates the low levels of gp120 and the variation among HIV-1 primary isolates. Isolates could be divided into three groups depending on the amount of virus-associated gp120. The primary isolates, R04, W49 and T04, had low virion gp120 content (<0.2ng/µU), similar to HIV-1\textsubscript{JRCSF}. The primary isolates, C29, Q19 and H44, had much higher levels of gp120 (0.7-1ng/µU), whereas the majority of isolates (5 of 11) fell into a group with intermediate levels of gp120 of around 0.5ng/µU.
Figure 4.1. The concentration of gp120 on laboratory-adapted and primary HIV-1 isolates.

A

![Graph showing gp120 concentration for different isolates]

**Isolate name**

- MN (P)
- NL4-3 (P)
- JRCSF (P)
- Y65
- D71
- W69
- T01
- O19
- R04
- H44
- M04
- C29
- J98
- X20

B

**Isolate name**

- MN (P)
- JRCSF (P)
- H44
- C29
- Q19
- T01
- Y65
- D71
- M04
- X20
- R04
- W69
- T01

**Figure 4.1.** Laboratory-adapted isolates of HIV-1, MN and NL4-3, were propagated in PBMCs (indicated by (P) after isolate name) or the H9 cell line (H9 after isolate name) over 14 days, when the cell supernatant was harvested. The CCR5-tropic laboratory-adapted HIV-1 strain, JRCSF, and all primary isolates were isolated from patients within 6 months of infection and propagated in PBMCs (Materials and Methods, section 2.6). Intact HIV-1 virions were purified and the gp120 content of viral pellets determined by ELISA (section 2.14). All results were normalised to RT activity. Uninfected cell supernatant constituted the negative control. **A.** virion-bound gp120 content of all isolates tested. **B.** expanded y-axis graph to reveal isolates with low virus-associated gp120. Error bars represent the standard deviation of triplicate ELISA samples from a single culture. Relevant $p$ values between groups of primary isolates are indicated with brackets, *** = $p < 0.001$. 
One isolate, J98 (Figure 4.1A), had significantly higher envelope gp120 than all the other primary isolates ($p<0.001$), with levels of gp120 equivalent to that of MN grown in H9 cells.

To confirm these findings, gp120 expression was investigated by Western blot (Figure 4.2). A significant difference in the amount of gp120 between MN grown in H9 cells or PBMCs was observed, again with the latter exhibiting lower amounts of glycoprotein. Smaller amounts of gp120 were also observed for HIV-1JRCSF and the three primary isolates tested compared to HIV-1MN grown in both H9 cells and PBMCs. It can be seen that normalising virus to RT activity does not correlate with equalising capsid protein in isolates, as bands corresponding to p24 varied widely. This is because quantification of RT activity excludes replication-defective virions from which p24 can still be detected.

**Analysis of the gp41 sequences of 4 primary isolates**

In order to assess whether the differences seen in gp120 content among primary isolates resulted from differences in amino acid sequence, gp41 from four of the primary isolates (X20, C29, M04 and J98) were amplified by PCR and sequenced (Materials and Methods, sections 2.20-2.23). The amino acid sequence of the N’-terminus of gp41 was determined, as this is one of the areas of gp41 that interacts with gp120 on the surface of the virion (Helseth et al., 1991; Poumbourios et al., 1995; Wyatt et al., 1998; Kao et al., 2001; York et al., 2004; Kim et al., 2008). Alterations in the amino acid sequence in this zone could, therefore, account for differences in the amount of gp120 per virion. Alterations in the amino acid code could lead to an increase in gp120 shedding and reduce the detection of gp120 in the ELISA assay used.

Sequencing was carried out to identify genetic differences in the transmembrane protein of J98 in particular, as this isolate possessed unusual amounts of gp120 in comparison to the all other primary isolates tested (Figure 4.1). On aligning the gp41 sequences of these primary isolates, with laboratory-adapted and primary HIV-1 strains of diverse clades from the Los Alamos HIV database (Figure 4.3), Isolate J had a unique sequence at position 667-670 which may contribute to the high levels of associated gp120.
Figure 4.2. Western blot analysis of HIV-1 envelope gp120 and p24 core protein.

Figure 4.2. Whole HIV-1 MN virions (propagated in PBMCs or H9 cells) and three primary HIV-1 isolates (H44, W49 and Y65) cultured from HIV-infected blood samples were purified, as described (legend for Figure 4.1). Viral pellets were normalised to RT activity and subjected to SDS-PAGE electrophoresis (Materials and Methods, sections 2.12 and 2.15, respectively). Separated proteins were blotted onto nitrocellulose membrane and gp120 and p24 proteins detected using rabbit polyclonal gp120 antiserum and mouse monoclonal p24 antiserum, respectively (section 2.15). Uninfected cell supernatant was the negative control and positive gp120 controls consisted of recombinant HIV-1IIIB gp120.
Figure 4.3. Analysis of HIV-1 gp41 sequences.

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Figure 4.3. Amino acid alignment of gp41 (amino acids 521-780) from laboratory-adapted and primary HIV-1 isolates. Viral gp41 N-terminal DNA sequences from four of the primary isolates (X20, C29, M04 and J98) tested for gp120 content were amplified by PCR and sequenced, as detailed in sections 2.22 – 2.23. DNA sequences were then translated into an amino acid sequence and aligned using the Multalign online server with sequences from laboratory-adapted (MN, HXB-2, NL4-3 and JRCSF) and other primary isolates of diverse clades (from diverse clades represented by the letter in their name) from the Los Alamos HIV database. Isolates are identified in the diagram by the first letter of their name (X, C, M and J).
observed with this isolate. The area of most diversity amongst all the sequences aligned was between position 663-670. Further investigation will be needed to determine the impact these differences in sequence have on virion-associated gp120.

**Comparison of envelope protein, gp120, on primary virus isolated at different points during in vivo infection.**

Three HIV-1 primary isolates derived from blood samples taken from HIV-infected individuals were assayed for their gp120 at two time points. Blood samples were taken shortly after infection (week 0) and one year later (week 52) and virus was isolated by co-culture of the patient PBMCs with those from HIV-1 negative donors. Patients were identified at primary infection, having had a negative HIV-1 serology test within six months (Materials and Methods, section 2.6). Secondary cultures were propagated by co-culturing primary cells with fresh PBMCs in order to stimulate virus replication and increase titre. The amount of virion-bound gp120 was then quantified as previously described (section 2.14). Figure 4.4 shows that two of the three isolates (E45 and W49) had significantly \( p<0.05 \) less gp120 one year after primary infection. The isolate, H44, exhibited no change in gp120 content between the two time points (Figure 4.3).

It may be noted that the amount of virus-associated gp120 on the H44 isolate in Figure 4.1B and Figure 4.4 is different. This was most likely due to the use of different batches of inoculum when repeating the culture of this isolate to produce virus for the gp120 ELISA.
Figure 4.4. gp120 content of three primary HIV-1 isolates.

Figure 4.4. Primary HIV-1 was isolated one year apart (week 0 and week 52), from three individual patients, H44, E45 and W49. Virions were purified from infected cell supernatant taken from secondary cultures and the virion pellets resuspended in PBS containing 0.5% Triton X-100 non-ionic detergent and the gp120 content determined by antigen capture ELISA (Materials and Methods, section 2.14). All results were normalised for RT activity and uninfected cell supernatant was included as the negative control. Error bars represent the standard deviation of triplicate ELISA samples from secondary cultures isolated at different times during infection. Relevant $p$ values between the two time points are indicated, * $= p < 0.05$. 

Isolate Name
- H44
- E45
- W49
Envelope cholesterol depletion of an HIV-1 primary isolate.

An HIV-1 B-clade primary isolate Env, T02, was pseudotyped. The pseudotyped virus produced to high titres in 293T cells by transfection of an HXB2 Δenv virus backbone along with a second plasmid containing the T02 isolate gp160 sequence (Materials and Methods, section 2.7) was used to explore antigenicity of primary HIV-1 Env after cholesterol depletion.

Virus-containing transfected cell supernatant was subjected to 1mM MBCD treatment and purified through a 20% sucrose cushion by ultracentrifugation (section 2.8). The viral cholesterol content was quantified using a fluorometric assay (section 2.9) and the percentage cholesterol calculated compared to an untreated control. Viral infectivity was assessed by titration onto the TZM cell line, a HeLa cell line, stably transfected with the β-galactasidase enzyme under the control of the HIV-1 LTR leader sequence (Kimpton et al., 1992). The β-gal enzyme is expressed as a result of Tat transactivation on HIV-1 infection of the cell and, after incubation with a β-gal substrate, infected cells appeared blue. The percentage residual infectivity post-cholesterol removal was calculated in comparison to an untreated control. Table 4.1 shows that MBCD treatment removed 40% of the envelope cholesterol, resulting in a 60% decrease in virus infectivity; findings that correlate with those found for HIV-1MN in Chapter 3 (Figure 3.1 and 3.2).

To assess the impact of cholesterol depletion on the primary pseudotyped HIV-1 protein content, RT activity was assayed by qRT-PCR, while p24 and gp120 levels were determined by ELISA. Table 4.1 shows that reductions were observed in both RT activity (40%) and in the p24 (25%) viral content. However, these changes were not confirmed by Western blot (Figure 4.5A and B), probably due to the lower sensitivity of this technique. Viral gp120 content (Table 4.1) was unaffected by cholesterol depletion, when assayed by ELISA, and was confirmed by Western blot (Figure 4.5A). No changes were recorded in expression of viral IN (p31) or unproccessed Gag (p55), detected using pooled HIV-1 positive patient sera (Figure 4.5B).
Table 4.1. Cholesterol content, protein composition and infectivity of pseudotyped HIV-1 post-cholesterol depletion.

<table>
<thead>
<tr>
<th>MBCD (mM)</th>
<th>Cholesterol (%)</th>
<th>Infectivity (FFU)</th>
<th>RT activity (µU per 5µl)</th>
<th>p24 (ng/ml)</th>
<th>gp120 (ng/ml)</th>
</tr>
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<tr>
<td>0</td>
<td>100</td>
<td>11.61</td>
<td>33.49</td>
<td>27.19</td>
<td>7.26</td>
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<td></td>
<td>+/- 2.20</td>
<td>+/- 0.10</td>
<td>+/- 0.46</td>
<td>+/- 0.10</td>
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<tr>
<td>1</td>
<td>56.73</td>
<td>4.31</td>
<td>20.43</td>
<td>13.29</td>
<td>7.83</td>
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<tr>
<td></td>
<td>+/- 3.67</td>
<td>+/- 0.53</td>
<td>+/- 0.73</td>
<td>+/- 0.10</td>
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Table 4.1. The envelope gene for a B-clade primary isolate, T02, was engineered into the HXB2 laboratory-adapted strain backbone to produce an HIV-1 pseudotype (Materials and Methods, section 2.7). Virus-containing cell supernatant was treated with 1mM MBCD for 1 hour at 37°C and purified as before (section 2.8). The control was untreated virus. Table 4.1 shows changes in viral envelope cholesterol (% residual cholesterol), infectivity (FFU), RT activity (µU per 5µl), p24 (ng/ml) and gp120 (ng/ml). Assays used to quantify all variables are detailed (sections 2.8 and 2.11 – 2.14). Table shows combined means +/- the standard error calculated from three independent experiments carried out in triplicate.
Figure 4.5. Western blots show the protein content of 1mM MBCD-treated virus (+) compared to the untreated control (-). Viral proteins were normalised to RT activity (Materials and Methods, section 2.12). **A.** gp120 was detected using rabbit gp120 antiserum, whilst gp41 and p24 were detected using mouse monoclonal antibodies. Secondary anti-rabbit and anti-mouse HRP-conjugated antibodies were used, respectively. **B.** pooled HIV-1 patient antisera with secondary goat anti-human conjugated to HRP enabled the detection other viral proteins. Details of antibody concentrations can be found in Materials and Methods, section 2.15. Uninfected cell supernatant comprised the negative control (negative) and p24 staining indicated equal loading of virus.
Effect of cholesterol depletion on primary HIV-1 neutralisation.

Neutralisation of a B-clade pseudotyped primary isolate, with or without viral envelope cholesterol removal, was assessed using the same panel of neutralising MAbs (2F5, 2G12, b12, 4E10 and 4.8D) used in Chapter 3. Changes in viral neutralisation by sCD4 and by homologous or three different heterologous HIV-1 patient antisera was also employed to monitor exposure of viral epitopes after cholesterol removal. Percentage infectivity inhibition was quantified using the TZM cell neutralisation assay, described in Materials and Methods (section 2.24) and calculated against virus incubated without MAb, sCD4 or antiserum.

The effect of cholesterol depletion of the HIV-1 primary pseudotype on neutralisation by MAbs was less than that observed previously with HIV-1MN (Chapter 3). No significant change in neutralisation was detected following cholesterol removal using the MAb 2F5 at 0.5µg/ml and 2µg/ml concentrations (Figure 4.6A). However, at a 2F5 concentration of 1µg/ml a 25% decrease in viral neutralisation was observed after cholesterol removal compared to the untreated control. Why an increase in neutralisation was seen at this concentration in particular is unclear, but may involve the binding dynamics of this antibody to its gp41 MPER epitope at this concentration.

With the MAb 4E10 (Figure 4.6B), which also binds to an MPER epitope in gp41, cholesterol depletion resulted in an increase in virus neutralisation, suggesting exposure of this epitope which is close to the viral membrane. Cholesterol removal from the virus increased the percentage inhibition of infectivity by 14%, 15% and 40% at concentrations of 0.5µg/ml, 1µg/ml and 2µg/ml, respectively.

There was no change in neutralisation of native or cholesterol-depleted virus in the presence of MAbs 2G12 or b12 (data not shown). This was due to a lack of MAb neutralising ability against this particular clade B primary isolate.

Unique data were obtained using the MAb 4.8D. Neutralisation by 4.8D was significantly ($p<0.05$) decreased after cholesterol depletion compared to an untreated control by 16% at a 4.8D concentration of 0.5µg/ml and 47% at a MAb concentration.
Figure 4.6. Effect of cholesterol depletion on neutralisation of an HIV-1 pseudotype by a MAb’s.

A

B

C

Figure 4.6. The pseudotyped B-clade HIV-1 isolate was treated with 1mM MBCD (dotted line), purified as described (section 2.8), and viral neutralisation assayed (Materials and Methods, section 2.24). The neutralising MAb’s, 2F5 (A), 4E10 (B) and 4.8D (C) were pre-incubated with virus for 1 hour at 37°C in a range of concentrations (x-axis). In all neutralisation experiments, percentage inhibition of infectivity (y-axis) was calculated using samples incubated without MAb. Untreated virus acted as the control (solid line). Error bars display the standard deviation of the mean calculated from two independent experiments carried out in triplicate. Relevant p values for cholesterol-depleted virus compared to the untreated control are indicated, * = p < 0.05 and ** = p < 0.01.
of 2µg/ml. In contrast, there was no change in neutralisation by 4.8D at a 1µg/ml concentration after removing cholesterol. The reduction in neutralisation sensitivity after cholesterol depletion of T02 with the MAb 4.8D (Figure 4.6C) could indicate masking of this epitope upon removal of cholesterol from the viral envelope.

Cholesterol depletion of pseudotyped HIV-1 isolate Env, T02, did not improve sCD4 neutralisation at concentrations of 0.5µg/ml or 2µg/ml tested. A 10% increase was observed at a sCD4 concentration of 1µg/ml, however, this was not statistically significant (Figure 4.7).

Homologous and three different heterologous HIV-1 patient antisera were assayed for neutralisation of both untreated and 1mM MBCD-treated virus (Figure 4.8). No change in viral neutralisation by homologous serum was observed after cholesterol removal. A statistically significant difference ($p<0.05$) in neutralisation after cholesterol depletion of the pseudotyped primary isolate was only observed using one of the three heterologous sera (heterologous serum 1, Figure 4.8), with a 40% increase in percentage inhibition of infectivity compared to the untreated control.
Figure 4.7. Effect of cholesterol depletion on the neutralisation of HIV-1 pseudotype by sCD4.

Figure 4.7. Pseudotyped isolate, T02, was treated with 1mM MBCD (dotted line) and purified, as described in the legend for Table 4.1 (page 159). Neutralisation of the virus by increasing concentrations of sCD4 (x-axis) was assayed using the TZM neutralisation assay, described in Materials and Methods (section 2.24). Untreated virus constituted the control (solid line). Inhibition is shown as a percentage of virus incubated without sCD4 (y-axis). Error bars display the standard deviation of the mean calculated from two independent experiments carried out in triplicate.
Figure 4.8. Effect of cholesterol depletion on the neutralisation of an HIV-1 pseudotype by HIV-1 patient antisera.

![Graph showing effect of cholesterol depletion on neutralisation](image)

**Figure 4.8.** Pseudotyped B-clade HIV-1, T02, was treated with MBCD (dotted bars) and purified, as described in the legend for Table 4.1 (page 159). Neutralisation of the virus by homologous and three different heterologous HIV-1 patient antisera (1-3) (x-axis), at a 1/20 dilution, was assayed using the TZM neutralisation assay (Materials and Methods, section 2.24). Inhibition is shown as a percentage compared to virus incubated without antisera (y-axis). Untreated virus (black bars) acted as the control. Error bars display the standard deviation of the mean calculated from two independent experiments carried out in triplicate. Relevant p values comparing cholesterol-depleted and untreated virus are indicated, * = p < 0.05.
4.3 Discussion

The HIV-1 laboratory strains, MN, NL4-3 and JRCSF were assed for gp120 content, as were 12 HIV-1 primary isolates. Those laboratory strains which were CXCR4 coreceptor using had the highest amount of virion-associated gp120. Laboratory-adapted viruses were grown in H9 cells, a T-cell line in which they replicate optimally, whilst primary isolates were grown in primary cells. For direct comparison, HIV-1\textsubscript{NL4-3} and HIV-1\textsubscript{MN} were also propagated in PBMCs. In doing so, significantly less surface gp120 was detected, compared to virus cultured in H9 cells. This could be due to differences in viral replication between cell types that affects virion-associated gp120 (Hammonds et al., 2003). It has been shown that pseudotyped HIV-1\textsubscript{NL4-3}, produced by transfection in 293T cells, contains more virus-associated gp120 than virus grown in H9 cells (Hammonds et al., 2003). Therefore, as observed for the growth of HIV-1\textsubscript{MN} in primary cells compared to T cell lines, the mode of virus production can affect virion-bound gp120 content (Hammonds et al., 2003).

These differences in HIV-1 gp120 content when the virus is grown in different cell lines could result from changes in gp160 cell surface expression. This could be confirmed by analysis of gp160 at the infected-cell surface, by Western blot or Fluorescence Activated Cell Sorting (FACS). Alternatively, if equal cellular expression of gp160 was found, differences in virus-associated gp120 could be linked to events after viral budding, for example gp120 shedding.

Among the CCR5-tropic isolates tested, there was considerable variation in viral gp120 content (ranging from 0.05-6.93ng of gp120 normalised to RT activity). The HIV-1\textsubscript{JRCSF} strain propagated in PBMCs was used as a laboratory-adapted HIV-1 comparison for primary isolates because it utilises the CCR5 co-receptor. This was, therefore, a more appropriate reference strain than the CXCR4-restricted strains propagated in cell lines that cannot support HIV-1 primary isolate replication. All primary isolates and the HIV-1\textsubscript{JRCSF} strain had much reduced levels of virion-bound gp120 compared to the CXCR4-restricted HIV-1 strains. The isolate, J98, was unusual among primary isolates in that it had comparable levels of gp120 to that on HIV-1\textsubscript{MN} grown in H9 cells or HIV-1\textsubscript{NL4-3} grown in primary cells. This illustrates that, despite the general rule that primary
isolates express less gp120, there are exceptions and these may be important for exploitation in vaccinology (Hammonds et al., 2003).

In two of three primary viruses sequentially isolated from the same patient one year apart, there was a decrease in virus-associated gp120 content over time. This may be due to genetic factors as the virus population in infected individuals a year after infection is not homogenous. The changes in gp120 observed over time could be due to selection of virus with reduced surface glycoprotein that could help the virus evade immune attack, although, no evidence of this has been reported in vivo.

Previous studies quantifying gp120 have used p24 capsid protein present in preparations to normalise virus sample content (McKeating et al., 1991; Williams et al., 1991; Chertova et al., 2002). As shown by Western blot (Figure 4.2), isolates normalised to RT activity illustrated wildly differing amounts of capsid protein, therefore, p24 levels may vary in different isolates. Similarly, normalising to RT activity does not take account of defective virions which also express gp120. Without being able to quantify accurately the number of viral particles, robust analysis of the amount of virion-bound envelope glycoprotein is problematic. Topographic electron microscopy which allows a 3-D image of individual virions to be created, can be used to calculate the number of gp120 spikes for a single virion without the hinderance of normalising the amount of virus present (Zanetti et al., 2006; Zhu et al., 2006).

Despite the fact that gp120 shedding limits viral infectivity (Schneider et al. 1986; McKeating et al. 1991), release of virus-associated glycoprotein benefits viral survival by sequestering antibodies produced by humoral immune responses (McKeating et al., 1991). However, low levels of gp120 on primary isolates (Karlsson et al., 1996) and the masked nature of HIV-1 glycoprotein epitopes is likely to be more significant than gp120 shedding in vivo (Moore et al., 1992; Wyatt et al., 1998).

Recombinant sCD4 consists of the extracellular domain of the CD4 receptor from the outer surface of the cell and has been widely used to induce gp120 shedding (Hart et al., 1991; Moore et al., 1992; Sattentau et al., 1993; Groenink et al., 1995; Chertova et al., 2002). However, sCD4 is not a true mimic for the viral receptor (Allan, 1991) and investigation of gp120 shedding induced by full-length and membrane-bound CD4
receptors, would be informative. For example, if gp120 shedding is found to be induced with membrane anchored, full length CD4 as has been shown with sCD4 (Moore et al., 1992; Sattentau et al., 1993; Chertova et al., 2002) this may have a significant impact on viral fusion and infectivity.

Laboratory-adapted HIV-1 strains readily shed virus-associated gp120 compared to primary HIV-1 isolates, a possible consequence of adaptation to growth in T cell lines (Schneider et al., 1986; McKeating et al., 1991; Moore et al., 1992; Groenink et al., 1995; Karlsson et al., 1996; Chertova et al., 2002). Uncleaved p55 creates glycoprotein molecules with substantially greater stability (Hammonds et al., 2003), indicating that the strength of the gp120:gp41 association may be critical in gp120 shedding. Mutations in the C-terminus of gp41, close to the site of gp120 interaction, has shown no link to viral gp120 shedding (Affranchino et al., 2006). Therefore, mutations that affect gp120:gp41 stability may be crucial in understanding glycoprotein shedding.

The gp41 sequences of primary isolates detailed in the present study did not show any significant genetic differences that could lead to the differences in virus gp120 content observed. Despite this, several mutational studies have lead to the hypothesis that gp120 shedding is a result of genetic differences between virus strains. Point mutations in HIV-1_HXB2 gp120 conferred instability in the gp120:gp41 association in 7 mutants (substitutions S528T, M530S, Q552L, L555G, E584A, V608S and W628M). As a result, cell-associated gp160 was reduced, but viral glycoprotein shedding was similar to wild type. These mutants were unable to induce syncytia and their infectivity was decreased due to the effect of gp120 mutation on protein function during fusion (Cao et al., 1993). In the same study, the mutation L669P (leucine to proline) was associated with increased cellular expression of gp160, but without changing virion gp120 incorporation or shedding. These data highlighted hydrophobic stretches in the C1, C3, C4 and C5 domains of gp120 that are important for the gp120:gp41 interaction (Cao et al., 1993) and show genetic changes may influence cellular expression of gp120 that have no affect on gp120 shedding.

Other mutational studies have highlighted possible links between glycoprotein sequence and shedding of virus-associated gp120. An A55F (alanine to phenylalanine) mutation in HIV-1_{IIB} gp120 ablated sCD4-induced shedding as a result of changes to the CD4
binding site that affected the efficiency of sCD4 binding (Hart et al., 1991). Substitution of HIV-1_{NL4-3} amino acid position 267, from glutamine to asparagine, reduced infectivity and increased the release of cellular gp120 into the medium. However, shedding of gp120 from viral particles remained unaffected (Willey et al., 1993).

Mutational analysis of retroviruses, other than HIV-1, has provided more information into the genetic factors at the route of surface glycoprotein shedding. A mutation at amino acid position 493, from lysine to valine, in Moloney murine leukaemia virus (MMuLV) resulted in reduced surface glycoprotein shedding from the virion. Sequential deletion of the cytoplasmic tail and transmembrane region of gp41 led to increased shedding of viral surface glycoproteins. The removal of the entire membrane spanning domain resulted in extensive shedding (Aguilar et al., 2003). These results indicate that genetic sequence and the presence of the cytoplasmic tail and hydrophobic membrane spanning region of the transmembrane glycoprotein may be important in reducing surface glycoprotein shedding.

A strain of SIV, CP-MAC, isolated from long-term culture also illustrated stability of the gp120:gp41 interaction with increased gp120 shedding (LaBranche et al., 1994; LaBranche et al., 1995). A tyrosine to cysteine mutation at amino acid residue 723 was responsible for the increased shedding of CP-MAC. Immuno-electron microscopy indicated that this mutant had a pattern of gp120 cellular expression that was evenly distributed across the cell surface, which is different to discreet patches of glycoprotein observed in the wild type (LaBranche et al., 1994; LaBranche et al., 1995). Despite this pattern of gp120 cellular expression, virion incorporation of gp120 remained unaffected (LaBranche et al., 1994; LaBranche et al., 1995).

In order to explore ways of increasing neutralisation of HIV-1 primary isolates with a view to vaccine development, the pseudotyped B-clade primary isolate, T02, was subjected to cholesterol depletion. Treatment of T02 with 1mM MBCD reduced the viral envelope cholesterol by 40% and, consequently, viral infectivity. Unlike HIV-1_{MN} (Chapter 3), isolate T02 did suffer loss of one third of viral RT activity and one fifth of the p24 content at this level of cholesterol depletion. Importantly, glycoprotein content was unaffected by the reduction in envelope cholesterol levels. These differences in
protein content with cholesterol depletion may be reflected in creating virus by transfection, compared to natural infection (for example, HIV-1\textsubscript{MN}, Chapter 3).

On analysing the neutralisation of the pseudotype, T02, by MAbs, sCD4 and homologous or heterologous sera, increases in neutralisation were observed with fewer neutralising MAbs compared with results obtained with HIV-1\textsubscript{MN} (Chapter 3). This reflects the problem that epitopes of primary isolate glycoproteins are highly masked (Wyatt \textit{et al.}, 1998). Among the MAbs tested, virus neutralisation was only enhanced post-cholesterol depletion with the MAb 4E10 and one heterologous HIV-1 patient antisera. Of the possible epitopes exposed in primary isolates, it is most likely those epitopes that are in close proximity to the viral envelope, like 4E10.

Cholesterol removal appeared to decrease the neutralisation capacity of the antibody 4.8D, highlighting that any changes in epitope exposure or conformation resulting from cholesterol depletion are just as likely to impact on viral neutralisation in a negative way. Thus, these data show that cholesterol depletion can alter the antigenicity of primary HIV-1 isolates, by exposing epitopes close to the viral membrane, or by changing protein conformation to hinder viral neutralisation.

Fewer changes in neutralisation were observed for the pseudotyped primary isolate compared to that observed with HIV-1\textsubscript{MN} in Chapter 3. This raises the possibility that cholesterol depletion alone may not be sufficient to elicit an enhanced immune response against HIV-1. Molecular pseudotyped viruses contain only one cloned envelope and this alone could account for the reduced effect of cholesterol depletion on increasing neutralisation. One advantage of using pseudotyped viruses is that they express stable gp120 at the virion surface (Hammonds \textit{et al.}, 2003). If wild-type primary isolates were used, a diverse array of different quasispecies would increase the potential of removing cholesterol as a means for enhancing the HIV-1 humoral response on vaccination. However, it is feasible that extending viral diversity by using a cocktail of pseudotyped virions from different clades could represent a vaccine tailored to worldwide viral diversity. This would not only incorporate a number of HIV-1 clades but also diverse gp120 clones within each clade.
The characteristics of HIV-1 laboratory-adapted isolates cannot be extrapolated to primary HIV-1 isolates for a variety of reasons, not least because of profound differences in antigenic structure (Sheppard, 2005), and the level of virus-associated gp120 (Race et al., 1995; Poon et al., 2005; Poon et al., 2005; Watson et al., 2009). Therefore, vaccine design based on HIV-1 primary isolates would represent the best presentation of world-wide circulating virus to the immune system. This would optimise the chance of eliciting a protective immune response by using relevant viral strains prevalent in different areas of the globe.

There are issues surrounding production of a primary isolate whole-inactivated HIV-1 vaccine, as virions typically possess small amounts of gp120. As the target immunogen for an HIV-1 vaccine is gp160, careful selection of isolates is key to optimise the amount of glycoprotein. To acquire 1µg of gp120, 1 litre of a typical titre (10^{5-6} infectious titre per ml) PMBC cell culture would be required (Hammonds et al., 2003). The amount of gp120 conventionally used in mouse or rabbit HIV-1 vaccine models is at least 1µg of gp120 per dose indicating the limitations of using primary isolates in vaccine studies (Borrow et al., 1994).

However, the use of pseudotyped isolates would facilitate presentation of primary isolate glycoproteins in virus that can be produced to high titre. Moreover, pseudotyped isolates produced by transfection retain virion-bound gp120 effectively (Hammonds et al., 2003). The screening of a panel of pseudotyped primary isolates for neutralisation sensitive clones has allowed the isolation of a clade B, primary isolate which is neutralised by the MAbs b12, 2G12 and 2F5 with more than an 80% decrease in viral infectivity (Lai, 2009). The isolation of a neutralisation sensitive isolate with the ability to retain gp120 would be a significant step forward in the application of primary isolates to HIV-1 vaccine research. Identification of such an isolate could then be used to investigate the effect of cholesterol depletion on the production of humoral responses in vivo.

In this chapter, we have shown that virion-bound gp120 varies widely among primary isolates, and laboratory-adapted HIV-1 strains grown in different cell types (Schneider et al., 1986; McKeating et al., 1991; Layne et al., 1992; Moore et al., 1992; Groenink et al., 1995; Karlsson et al., 1996; Chertova et al., 2002; Hammonds et al., 2003).
Therefore by extension, a panel of HIV-1 primary isolates that possess high levels of gp120 could be established. The level of virus-associated gp120 may also be affected by stage of infection, although more isolates taken at different stages of infection would need to be studied to confirm this.

Cholesterol depletion of a pseudotyped primary isolate can alter the conformation of virus-associated gp160 and enhance epitope exposure of primary HIV-1. Cholesterol depletion could, therefore, enhance HIV-1 primary isolate whole-inactivated vaccines. Combined with the incorporation of diverse quasispecies and clades of HIV-1, best representing field isolate diversity, this could represent an attractive potential HIV-1 immunogen.
Chapter 5:

Chemical inactivation of HIV-1 and preservation of gp160 antigenicity.
Inactivated vaccines have been successfully used to prevent disease, as evidenced by the Salk Polio vaccine (Salk et al., 1960) and Hepatitis A inactivated vaccines (Werzberger et al., 1992; Clemens et al., 1995; Werzberger et al., 1998). Three major problems are associated with whole-inactivated HIV-1 vaccines. Firstly, the presence of residual infectious virus as a result of incomplete inactivation is a substantial risk (Dudani et al., 2008). Secondly, ensuring retention of the key immunogenic epitopes post-inactivation is important in mounting a protective immune response. For example, chemical inactivating agents may detrimentally affect protein immunogen conformation and produce an ineffective immunogen, as has been the case with HIV-1 (Sattentau, 1995; Grovit-Ferbas et al., 2000). Finally, the use of inactivating agents that are toxic require removal by purification, steps which could affect the conformation of the viral envelope proteins (Dudani et al., 2008).

This chapter investigates the efficiency of inactivating agents with different mechanisms of action. These agents were assessed for their potential to inactivate cholesterol-depleted HIV-1, while retaining the integrity of gp120, with the ultimate aim of exploring the potential use of whole-inactivated HIV-1 as a candidate vaccine. Four inactivating agents were used for this purpose. These included two agents that non-specifically crosslink all viral proteins (formalin and BPL) and two that mediate viral inactivation by specifically acting on individual HIV-1 proteins (AT-2 and NEM), for example, AT-2 targets the zinc finger motifs within the NC.

*In vitro* studies have shown that formalin inactivates biological agents by cross-linking nitrogen atoms in proteins, particularly in lysine residues and peptide bonds, to form methylene bridges (−CH$_2$−) (Hopwood, 1969). Although formalin efficiently inactivates HIV-1 and reduces infectivity by up to 7 logs (Grovit-Ferbas et al., 2000), maintenance of gp120 conformational and functional integrity following inactivation depends on the concentration of formalin used (Marx et al., 1986; Sattentau, 1995; Grovit-Ferbas et al., 2000).
Formalin inactivation is a first-order chemical reaction and, therefore, the rate of reduction in HIV-1 infectivity is constant. However, this rate is dependant on factors that include the temperature of inactivation, pH of the final solution and the concentration of formalin (Salk et al., 1960). The linearity of the reaction means that treatment parameters can be calculated so that a safety margin of error in reduction of viral infectivity can be accounted for. Thus, a concentration of formalin can be used to inactivate a virus sample containing up to 10,000 more TCID$_{50}$/ml than it actually contains (Salk et al., 1960).

A formalin concentration of 0.1% is known to destroy conformational epitopes in gp120 (Sattentau, 1995; Grovit-Ferbas et al., 2000). Therefore, lower amounts of formalin can be used over a longer incubation time in order to preserve antigenicity. Heat treatment combined with formalin concentrations of 0.02%, each of which alone fails to ablate viral infectivity, completely inactivates HIV-1 and preserves glycoprotein antigenicity (Sattentau, 1995; Grovit-Ferbas et al., 2000). Data published by Grovit-Ferbas et al. (2000) recorded up to a 7-log reduction in HIV-1 (isolates SX and NL4-3) infectivity when inactivation by 0.02% formalin was combined with 62°C heat treatment for 10 minutes. McDougal et al. (1985) reported that the sole use of 62°C heat treatment can inactivate HIV-1$_{LAV}$ at a rate of 1-log per 24 seconds. In this chapter, the combination of 0.02% formalin with either 45°C or 62°C heat treatment was investigated to determine whether lower concentrations of formalin combined with heat treatment, could maintain gp120 conformation maintenance and retain epitopes after cholesterol depletion, compared to 0.8% formalin.

The second protein cross-linking agent tested for its ability to inactivate HIV-1 was the alkylating agent, BPL. At low concentrations (0.2%), BPL can reduce HIV-1 infectivity in vitro by at least $10^5$ TCID$_{50}$ as it reacts with proteins, in particular lysine, cysteine and histidine amino acid residues to add alkyl groups (Race et al., 1995; Race et al., 1995). Utilised in the investigation of rabies and SARS vaccines (Jayakumar et al., 1990; Tang et al., 2004), BPL is safe for use in humans, as it is readily hydrolysed to β-hydroxypropionic acid, a non-toxic by-product of fat metabolism (Race et al., 1995).
More recent work involving HIV-1 inactivation has focused on an agent, AT-2, that targets the NC protein, due to the pivotal chaperone role of NC in reverse transcription, RNA packaging and, hence, HIV-1 replication (Section 1.X) (Rein et al., 1996; Morcock et al., 2005). The AT-2 compound can oxidise NC, creating the loss of a central chelated zinc ion leading to disulphide coupling of residues in this zinc finger region (Arthur et al., 1998; Lifson et al., 2002; Lifson et al., 2004). In vitro studies involving AT-2 inactivation of HIV-1MN and HIV-1LAI (this is the same isolate as HIV-1IIIB, first isolated by Barre-Sinoussi et al. (1983)), illustrated that viral infectivity can be reduced by up to 5 logs at concentrations of 1mM (Arthur et al., 1998). This was due to an inactive NC protein causing suspension of HIV-1 replication before reverse transcription and integration (Rice et al., 1997; Tummino et al., 1997). Inactivated virions can still fuse with the cell, suggesting that they remain intact and that surface glycoprotein function is preserved (Lifson et al., 2004).

A panel of mild hydrophobic oxidising agents like AT-2 (Arthur et al., 1998; Lifson et al., 2002; Lifson et al., 2004), dithiobis benzamide (DIBA) and 3-nitrosobenzamide (NOBA) has been investigated as potential anti-retroviral drugs (Miller Jenkins et al., 2005) and in HIV-1 inactivated vaccines (Rice et al., 1995; Rice et al., 1997; Miller Jenkins et al., 2005). These thioester compounds are capable of ejecting zinc from the carboxyl-terminal zinc finger domains of the NC protein and could, therefore, lead to disruption of viral infectivity (Miller Jenkins et al., 2005). Compounds such as these present promising candidates as potential new -antiretroviral drugs where non-toxic derivatives can be found.

Unlike AT-2, which acts by covalently linking viral proteins, NEM is an alkylating agent that reacts with thiol groups on cysteine residues to render proteins inactive without creating cross-linked oligomers (Morcock et al., 2005; Morcock et al., 2008). It is documented that NEM inactivates the RNase H activity of RT, but not RNA-dependant DNA polymerase activity due to the differences in the selective modification of amino acid residues between these two enzymes (Hizi et al., 1991; Morcock et al., 2005). A four-fold increase in concentration of NEM is required to equal AT-2 potency in HIV-1 inactivation (Morcock et al., 2005).
The aims of this chapter were to find a gentle, but effective inactivating agent that would not disrupt native glycoprotein conformation. Moreover, we wanted to demonstrate complete inactivation of cholesterol-depleted whole virus whilst retaining the unmasking of key epitopes that results from increasing the fluidity of the membrane into which gp160 is embedded. This was done in preparation for investigating the immunogenicity of whole-inactivated, cholesterol-depleted HIV-1 \textit{in vivo}.

The non-specific inactivating agents, formalin and BPL, as well as agents targeting individual HIV-1 proteins, NEM and AT-2, were tested on the HIV-1 laboratory strain, MN, with or without cholesterol depletion. Reduction in viral infectivity and cell-virus fusion was assessed by NP2 and C8166 titration assays. Virus was assayed post-inactivation by qRT-PCR to monitor RT activity, while gp120 and p24 protein composition was measured by ELISA. Preservation of surface glycoprotein conformation was investigated using an anti-gp120 antibody binding ELISA.
5.2 Results

Reduction of HIV-1 infectivity following chemical inactivation.

Initially, supernatant from HIV-1_{MN} infected cells was inactivated by formalin. Excess formalin was quenched by 0.2% BSA in PBS and intact virions were purified by ultracentrifugation through a 20% sucrose gradient. The viral pellet was resuspended in PBS and the infectivity assayed by titration on PBMCs, as described in Materials and Methods (section 2.10). Infectivity was reduced with cholesterol-depleted virus (1mM MBCD) compared to the untreated control, at days 7 and 14 (Figure 5.1), producing 15% less p24, which decreased to 50% at day 21. This is most likely due to decreased fusion ability, resulting from cholesterol depletion, lowering the level of infection.

A formalin concentration of 0.8% was chosen for initial inactivation experiments because this concentration effectively inactivates HIV-1, regardless of the effect on glycoprotein conformation and is useful for comparison with milder formalin concentrations. Inactivation of HIV-1 to below detectable levels was achieved using 0.8% formalin, both alone and combined with cholesterol depletion (Figure 5.1A). Although, 0.8% formalin effectively reduces viral infectivity, utilising lower concentrations of formalin would be more advantageous, as any adverse effects on glycoprotein conformation would be reduced. Therefore, 0.02% formalin, which is suboptimal for viral inactivation was chosen in order to preserve glycoprotein structure and was used in combination with 45°C or 60°C heat treatment (30 minute incubation) to destroy viral infectivity.

Abolition of HIV-1_{MN} infectivity was not achieved with 0.02% formalin combined with heat treatment at 45°C, as viable virus was detected by p24 production (~5%) after three weeks of incubation (Figure 5.1B). When this method of inactivation was combined with cholesterol depletion, no p24 production was observed (Figure 5.1B). Increasing the temperature, from 45°C to 62°C, together with 0.02% formalin reduced this residual infectivity. Although 0.02% formalin is suboptimal in ablating viral replication, when combined with cholesterol depletion and heat treatment at 62°C, this method of inactivation is effective.
Figure 5.1: Effect of inactivating agents on HIV-1 infectivity.

Figure 5.1. HIV-1MN (1 x 10^5 TCID<sub>50</sub>/ml) was inactivated with a variety of agents, with or without 1mM MBCD treatment (indicated in the key). Virus was inactivated by: A - 0.8% formalin, B - 0.02% formalin combined with 45°C or 62°C heat inactivation, C - 0.2% BPL, D - 1mM NEM or E - 1mM AT-2. Viral infectivity post-inactivation was assessed by titration on PBMCs and p24 monitored weekly over 21 days by ELISA (Materials and Methods, section 2.10). Error bars represent the standard deviation of triplicate samples from a single experiment.
The agent, BPL, inactivates HIV-1 by causing non-specific alkylation of viral proteins. Inactivation of HIV-1\textsubscript{MN} by 0.2% BPL alone or combined with cholesterol depletion (Figure 5.1C), ablates viral replication over 21 days. However, treatment of cholesterol-depleted virus using 0.2% BPL showed 30% residual p24 production at day 7. Detection of p24 in the cell supernatant early during the PBMC titration assay (e.g. day 7), may be due to residual p24 in the inoculum, however, as this was not demonstrated with all viral samples it is unlikely. Despite detection of p24 at Day 7, viral inactivation is best assessed over a period of three weeks. Titration experiments using PBMC’s were not run for longer than three weeks due to a reduction in the viability of the cells after this culture period.

Despite the alkylating agent, NEM, having a similar alkylation inactivation mechanism to BPL, it specifically targets cysteine residues within viral proteins (Morcock \textit{et al.}, 2005). Treatment of virus with NEM (Figure 5.1D), both alone and in combination with cholesterol depletion, reduced viral replication to undetectable levels after 21 days. At day 7, NEM combined with cholesterol depletion produced approximately 15% of the p24 produced by the untreated control virus, again this was most likely residual p24 from the inoculum.

The inactivating agent, AT-2, reacts predominantly with NC protein zinc fingers (Rein \textit{et al.}, 1996; Miller Jenkins \textit{et al.}, 2005; Morcock \textit{et al.}, 2008). Inactivation of HIV-1\textsubscript{MN} by AT-2 significantly decreased viral replication, but this was not abolished over 21 days. Production of p24, over 3 weeks, increased to 20% of the untreated control (Figure 5.1E) after titration of AT-2 inactivated virus onto PBMCs. When combined with cholesterol depletion, AT-2 inactivation reduced p24 production to below detectable levels, despite 10% residual p24 at day 7.

Fusion ability of inactivated virus was quantified using a C8166 syncytium induction assay (Materials and Methods, section 2.11). Table 5.1 shows that cholesterol depletion of HIV-1\textsubscript{MN} did not alter the ability of virus to fuse with cells. Inactivation of cholesterol-depleted HIV-1\textsubscript{MN} using 0.8% formalin, combined with cholesterol depletion, inhibited HIV-1\textsubscript{MN} cell-cell and viral-cell fusion. The same result was observed when cholesterol-depleted HIV-1\textsubscript{MN} was inactivated by 0.02% formalin
Table 5.1: Effect of inactivation of HIV-1 on fusion ability.

<table>
<thead>
<tr>
<th>Inactivation treatment</th>
<th>Fusion</th>
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<tbody>
<tr>
<td>None</td>
<td>+ + +</td>
</tr>
<tr>
<td>1 mM MBCD</td>
<td>+ + +</td>
</tr>
<tr>
<td>1 mM MBCD + 0.8% formalin</td>
<td>-</td>
</tr>
<tr>
<td>1 mM MBCD + 0.02% formalin + 45°C</td>
<td>-</td>
</tr>
<tr>
<td>1 mM MBCD + 0.02% formalin + 62°C</td>
<td>-</td>
</tr>
<tr>
<td>1 mM MBCD + 0.2% BPL</td>
<td>-</td>
</tr>
<tr>
<td>1 mM MBCD + 1 mM AT-2</td>
<td>-</td>
</tr>
<tr>
<td>1 mM MBCD + 1 mM NEM</td>
<td>+</td>
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</tbody>
</table>

Table 5.1. Inactivation of HIV-1_{MN} was carried out, as described in the legend for Figure 5.1. Viral infectivity and fusion ability post-inactivation were assessed using a syncytium induction assay on C8166 cells (Materials and Methods, section 2.11). Syncytia production was scored, + more than 5 syncytia, + + more than 15, + + + + more than 25 and - syncytia not observed.
combined with 45°C or 62°C heat treatment, 0.2% BPL or 1mM AT-2. The only inactivating agent which allowed retention of the fusion of HIV-1\textsubscript{MN} post-inactivation was NEM, however, viral fusion was significantly reduced.

**Effect of HIV-1 inactivation on RT activity**

Residual reverse transcriptase activity of inactivated virus was measured by qRT-PCR (Pizzato et al., 2008) immediately after inactivation and calculated as the percentage residual RT activity relative to control virus that was not inactivated.

As observed in Chapter 3, treatment of HIV-1\textsubscript{MN} with 1mM MBCD does not reduce RT activity (Figure 5.2A, labelled Control). The RT activity of HIV-1\textsubscript{MN} inactivated with 0.8% formalin (with or without cholesterol depletion) was dramatically reduced to less than 10% of the level in the untreated control (Figure 5.2A). Reverse transcriptase activity was less affected by lower concentration of formalin, 0.02%, where viral RT activity decreased by 60% using 0.02% formalin alone. However, for 0.02% formalin treatment, cholesterol depletion appeared to protect the virus from reductions in RT activity, with only a 10% loss post-inactivation (Figure 5.2A).

Reverse transcriptase activity of virus post-inactivation was lowered to 20% and 40%, respectively when treated with 0.2% BPL alone, or when combined with cholesterol depletion (Figure 5.2A). Statistical analysis (Mann-Whitney, non-parametric test) showed that this difference was not significant and, therefore, cholesterol depletion combined with BPL inactivation had no affect on RT activity. Similarly, when virus was inactivated using 1mM NEM alone or combined with cholesterol depletion, RT activity was reduced by 20% (Figure 5.2A).

RT activity fell sharply for cholesterol-depleted native virus when inactivated using AT-2. In these cases, a reduction of 60% was recorded.

In order to investigate the efficacy of 0.02% formalin, 45°C or 62°C heat treatment and cholesterol depletion on reducing viral RT activity, all possible combinations of these treatments were implemented to identify the role of each in reducing virus infectivity.
Figure 5.2: Effect of HIV-1 inactivation on RT activity.

Figure 5.2. RT activity (assayed by qRT-PCR) remaining immediately after inactivation was calculated as a percentage of the untreated control (labelled control). The HIV-1MN was subjected to inactivation with or without 1mM MBCD treatment. A, untreated virus (blue) or cholesterol-depleted virus (red) was subjected to inactivation using 0.8% formalin, 0.02% formalin, 0.2% BPL, 1mM NEM or 1mM AT-2, as described in Materials and Methods, section 2.25. Reverse transcriptase activity post-inactivation was also assessed using 0.02% formalin combined with B, 45°C heat treatment for 30 minutes or C, 62°C heat treatment for 30 minutes (section 2.25). Uninfected cell supernatant was the negative control. Error bars indicate standard deviation of the mean of a single experiment carried out in triplicate.
Immediately after inactivation of virus by 0.02% formalin alone (Figure 5.2A), viral RT activity decreased to 40% of the untreated control. Figure 5.2B shows that 45°C heat treatment of HIV-1\textsubscript{MN} combined with 0.02% formalin failed to reduce RT activity further indicating that addition of 45°C heat treatment provided no benefit. Inactivation of HIV-1\textsubscript{MN} by 45°C heat treatment alone only reduced RT activity by less than 10%. Figure 5.2B shows that the combination of cholesterol depletion with 45°C heat treatment did not reduce RT activity more than heat treatment alone. Inactivation using both 0.02% formalin and 45°C heat treatment combined with cholesterol depletion (Figure 5.2B) reduced viral RT activity by 20%. This reflects the loss of RT activity seen with cholesterol depletion after 0.02% formalin treatment described in Figure 5.2A. In summary, the combination of 45°C heat treatment with 0.02% formalin and envelope cholesterol removal does not in any way enhance inactivation, as assessed by losses in RT activity.

The higher temperature of 62°C, however, is more effective in enhancing inactivation of HIV-1\textsubscript{MN} by 0.02% formalin. Temperatures of 62°C alone for 30 minutes reduced RT activity by more than 90% of the untreated control (Figure 5.2C). This was confirmed by the observations that 62°C heat treatment combined with 0.02% formalin and cholesterol depletion with 62°C heat treatment could also reduce RT activity by more than 90%, higher than that recorded using 0.02% formalin alone (Figure 5.2A). Figure 5.2C shows that inactivation of cholesterol-depleted virus using 0.02% formalin combined with 62°C heat treatment effectively reduced RT activity to 15%.

The effect of HIV-1 inactivation on p24 content.

In order to assess the impact of viral inactivation on HIV-1\textsubscript{MN} core protein, p24 was assayed by ELISA after inactivation and purification of whole virus particles. Residual p24 was calculated and expressed as a percentage of the level determined for virus that was not inactivated (control). Uninfected cell supernatant constituted the negative control to measure non-specific binding within the ELISA.

No reduction in the p24 core protein was observed following cholesterol depletion of HIV-1\textsubscript{MN} by 1mM MBCD (Figure 5.3), thus confirming data generated in Chapter 3.
Figure 5.3: Effect of HIV-1 inactivation on p24 content.

Figure 5.3. HIV-1MN was subjected to inactivation alone (blue) or combined with 1mM MBCD cholesterol depletion (red). Total p24 content was quantified by ELISA (Materials and Methods, section 2.13) and percentage residual protein calculated relative to untreated virus (labelled control). Uninfected cell supernatant was the negative control. Error bars represent the standard deviation of the mean from a single experiment carried out in triplicate.
Virus inactivation by 0.8% formalin alone did not affect the p24 levels, but, when combined with cholesterol depletion a decrease of 20% was observed. This suggests that cholesterol depletion affected the permeability of viral particles so that on 0.8% formalin inactivation, p24 was lost from the virus.

Inactivation of HIV-1MN using 0.2% BPL (Figure 5.3), or when combined with cholesterol depletion gave the largest decrease in p24, whereby 40% of the viral capsid protein was lost compared to the untreated control. This is in contrast to treatment of HIV-1MN with AT-2 in conjunction with cholesterol depletion, where a 20% reduction in viral p24 content was observed.

Inactivation of cholesterol-depleted virus with lower concentrations of formalin (0.02%) combined with 45°C or 62°C heat treatment designed to retain glycoprotein antigenicity, maintained p24 levels post-inactivation, indicating preservation of intact virions (Figure 5.3). Assessment of native virus inactivated by 0.02% formalin, heat treatment or formalin combined with heat treatment was not carried out. Inactivation of virus using NEM alone or combined with cholesterol depletion only reduced p24 by 10% and 20%, respectively, suggesting that this method of inactivation also preserves intact virus.

**Effect of inactivation on HIV-1MN gp120 content.**

To assess the impact of chemical inactivation of HIV-1MN on glycoprotein content, gp120 was assayed by ELISA immediately post-inactivation. The percentage residual gp120 was calculated relative to an untreated HIV-1MN control. Any changes in gp120 conformation as a result of inactivation might influence the detection of envelope glycoprotein, therefore, a polyclonal antibody directed against a conserved sequence (APTKAKRRVQREKR) of the HIV-1 envelope, able to recognise denatured protein was used.

Contrary to the maintenance of viral gp120 content observed after cholesterol depletion in Chapter 3 (Figure 3.5), a 23% reduction in gp120 after 1mM MBCD treatment was observed (Figure 5.4). This was likely due to the incubation of virus at 37°C for twice
Figure 5.4: Effect of HIV-1 inactivation on gp120 content.

**Figure 5.4.** Supernatant taken from HIV-1<sub>MN</sub> chronically-infected cells was subjected to inactivation by the compounds indicated, alone (blue) or in combination with 1mM MBCD treatment (red). Uninfected cell supernatant was included as a negative control. Total gp120 content was quantified by ELISA (Materials and Methods, section 2.14) and percentage residual protein calculated against an untreated control. Error bars represent the standard deviation of the mean from a single experiment carried out in triplicate.
as long as in Chapter 3 in order to assess the combination of cholesterol-depletion and viral inactivation, which each require a 1 hour incubation step. Therefore, this longer incubation time may account for the increased loss of gp120, possibly resulting from gp120 shedding.

Inactivation of virus by 0.8% formalin, with or without cholesterol depletion, reduced virion gp120 content dramatically, to 7% of the untreated control (Figure 5.4). When cholesterol-depleted virus was treated with 0.02% formalin combined with heat treatment at 45°C or 62°C, the reduction in viral gp120 was temperature-dependent, as a 46% loss in gp120 was observed at 62°C whereas, only a 26% reduction was recorded at 45°C (Figure 5.4). The individual treatments of formalin, 45°C or 62°C and all combinations of these treatments were not tested, as it had been previously shown that gp120 is retained upon inactivation using this method (Grovit-Ferbas et al., 2000; Poon et al., 2005; Poon et al., 2005). However, it would be interesting, given the reductions in gp120 observed in this study to investigate these parameters. It can be assumed that the 26% reduction in HIV-1MN gp120 content when treated with 0.02% formalin and 45°C heat treatment was due to cholesterol depletion alone as observed for the control. However, the increase in heat treatment to use 62°C elicited a further 20% decrease in viral gp120.

Treatment with BPL resulted in little loss of viral gp120, however, when combined with cholesterol depletion, 60% of the virus surface glycoprotein was removed. Virion gp120 content decreased 29% when inactivated using 1mM NEM alone, but when gp120 was only reduced by 14% after cholesterol depletion and inactivation. Aldrithiol-2 inactivation caused the most significant loss in virion gp120 content, with an 79% reduction when cholesterol depletion was combined with inactivation. Inactivation of virus with AT-2 alone, however, only effected a 34% loss in viral gp120.

After correction for multiple comparisons using Bonferroni’s correction none of the differences observed in Figures 5.2 to 5.4 could be statistically proven as significant by non-parametric tests. Therefore, it is important to repeat these experiments to gain a robust picture of the effect of cholesterol depletion on RT activity, viral p24 and gp120 content post-inactivation.
**Preservation of viral glycoprotein conformation post-inactivation.**

The conformation of HIV-1 surface glycoprotein was investigated using infected cell supernatant inactivated with the agents described, followed by purification of whole viral particles. To assess gp120 structure, the conformation-dependent MAb, b12 (Burton et al., 1991; Barbas et al., 1992; Roben et al., 1994), was used in an HIV-1 binding ELISA (Materials and Methods, page X). Supernatant from uninfected cells constituted the negative control to detect non-specific antibody binding within the ELISA.

Inactivation of virus using 0.8% formalin destroyed the b12 conformational epitope on gp120 (Figure 5.5A). This is in part due to the loss of gp120 from the virion, although, concentrations of formalin at this level would most likely be enough to render gp120 conformationally non-reactive to antibody binding (Sattentau, 1995; Grovit-Ferbas et al., 2000).

Compared to this, inactivation of cholesterol-depleted virus by 0.02% formalin combined with 45°C heat treatment, only resulted in 50% reduction in MAb binding. This is mostly due to changes in protein structure alone, as gp120 content was only reduced by 20%. Similarly, when using 0.02% formalin and 62°C heat treatment, a 50% reduction in b12 binding indicated loss of glycoprotein antigenicity. These absorbance values are lower than would be expected due to the loss of gp120 alone, therefore, heat treatment at 62°C appears to compromise gp120 integrity.

Inactivation of cholesterol-depleted virus using both 1mM AT-2 and 0.2% BPL not only retained gp120 conformational epitopes (Figure 5.5A), but slightly enhanced antibody binding (borderline significance in the case of AT-2, \( p = 0.08 \)). Treatment of HIV-1\textsubscript{MN} using 1mM NEM after cholesterol depletion retained equal b12 binding compared to virus not subjected to inactivation.

In order to assess changes in antibody binding accurately, it is important to take into account gp120 loss as a result of inactivation. Figure 5.5B shows that with normalisation of antibody binding to residual gp120 content, small increases in antibody
**Figure 5.5:** Maintenance of HIV-1 gp120 conformation following cholesterol depletion and inactivation.

**A**

![Bar chart](image)

**B**

![Bar chart](image)

**Figure 5.5.** The conformation of HIV-1<sub>MN</sub> gp120 post-inactivation was determined by MAb binding ELISA (Materials and Methods, section 2.18) using the conformation-dependant MAb, b12. **A.** Viral samples, consisting of 100ng of p24, were lysed using 0.5% Triton X-100 in PBS and quantified by p24 ELISA (Materials and Methods, section 2.13). Uninfected cell supernatant acted as a negative control and was used to determine cut-off values. Error bars display standard deviation of the mean calculated from a single experiment carried out in triplicate. **B.** Absorbance values were normalised to gp120 due to the differences in viral gp120 content post-inactivation (Figure 5.4). Error bars represent standard deviation of the mean from a single experiment carried out in triplicate.
binding can be observed for cholesterol-depleted virus with or without inactivation by NEM. Virus cholesterol depletion combined with BPL or AT-2 treatment, elicited 5- and 6-fold increases in b12 binding, respectively. This enhancement of gp120 antigenicity beyond the levels observed with cholesterol depletion alone is intriguing. Grovit-Ferbas et al. (2000) have demonstrated a 2-fold increase in 17b binding to HIV-1SX after heat inactivation at 62°C. This suggests that inactivation processes alone may result in exposure of some viral epitopes.

After correcting results for multiple comparisons using Bonferroni’s correction, the differences observed could be proven statistically significant using a non-parametric test. Therefore, it is important to repeat these experiments to gain a robust picture of the effect of cholesterol depletion on RT activity post-inactivation.
5.3 Discussion

Formalin has been used in vaccine preparations for many years, the Salk vaccine being a prime example, as well as in development of SARS and rabies immunogens (Salk et al., 1960; Jayakumar et al., 1990; Tang et al., 2004). It is utilised not only as an inactivating agent, but also as a preservative in widely available vaccine formulations, such as the commercial inactivated polio vaccine Ipol (Aventis Pasteur) (Race et al., 1995). Most vaccines on the market are either inactivated or attenuated (see Appendix 1), illustrating that the inactivation technique is proven in the vaccine field.

Inactivation of HIV-1\textsubscript{MN} using 0.8\% formalin combined with cholesterol depletion efficiently destroyed virus infectivity to levels below detection (reducing infectivity by $10^5$ TCID\textsubscript{50}/ml). Despite only small reductions in viral p24 content post-inactivation, most envelope gp120 was lost and, hence, this method of inactivation would not be ideal for producing a whole-inactivated HIV-1 vaccine where surface glycoprotein is the target antigen. Compounded with this, antibody recognition of surface glycoprotein conformation was reduced, likely due to a combination of loss in gp120 protein and changes to protein epitope structure as a result of formalin treatment.

The antigenicity of HIV-1 is highly dependent on the native gp160 trimeric structure, whereas commercially available vaccines which utilise formalin as the inactivating agent do not have this restriction. This is because antigenicity is retained even with high levels of formalin treatment, as evidenced by the induction of effective immune responses \textit{in vivo} (Salk et al., 1960). This is most likely due to neutralising epitopes that do not require strict conformational presentation, unlike many HIV-1 gp160 epitopes.

The potential of combining lower concentrations of formalin with heat treatment, in order to preserve glycoprotein structure after cholesterol depletion and viral inactivation was investigated. Viral inactivation by 0.02\% formalin with 45\°C or 62\°C heat treatment carried out in this study, gave a lower inactivation potential compared to previously published data ($10^5$ TCID\textsubscript{50}/ml in this study compared to $10^{6.25}$ TCID\textsubscript{50}/ml in a study by Grovit-Ferbas et al. (2000)) (McDougal et al., 1985; Grovit-Ferbas et al., 2000). The lower inactivation potential observed in this study may be due to a number
of different reasons including the lower amount of input virus (10^5 TCID_{50}/ml) or strain used.

Despite there being no changes in viral RT activity or p24 content, gp120 was reduced by 40% using heat inactivation at 62°C combined with formalin treatment. However, when HIV-1 was inactivated by 0.02% formalin and 45°C heat treatment, gp120 content was reduced by only 20%. This confirms that gp120 loss is temperature-dependent (McKeating et al., 1991; Moore et al., 1992; Chertova et al., 2002).

Despite this evidence, a direct relationship between RT activity and viral infection has been described (Dimitrov et al., 1993). Therefore, taking into account these factors and, on the assumption that they are constant for individual virus strains, RT activity can be used as a marker for infectivity because without functional RT, retroviral replication cannot occur (Dimitrov et al., 1993).

Analysis of gp120 conformation using the antibody b12 showed that antibody binding was also temperature-dependent with greater loss of gp120 conformation at 62°C. This is contradictory to published data indicating that changes in gp120 content or conformation were not observed when the HIV-1_{SX} strain was subjected to 0.02% formalin combined with 60°C heat treatment (Sattentau, 1995; Grovit-Ferbas et al., 2000). Incubating virus at a temperature of 60°C has also been shown to have no effect on gp120 conformational epitopes (Grovit-Ferbas et al., 2000). However, MN not only has low amounts of virus-associated gp120, but it also tends to sheds glycoprotein more easily (McKeating et al., 1991; Moore et al., 1992; Hammonds et al., 2003). Therefore, in the case of HIV-1_{MN} loss of gp120 content as well as conformation may be expected.
It has been suggested that combining formalin treatment with heat inactivation in this way, can enhance both sCD4 and antibody binding (2G12, 17B and a V3 targeting antibody) to gp120 as a result of exposing viral epitopes. This has been shown in the HIV-1 laboratory-adapted strains SX and NL4-3 (Grovit-Ferbas et al., 2000), but highlights the potential for increasing HIV-1 antigenicity with heat treatment in vitro. This effect is strain-specific, and possibly linked to R5 co-receptor usage, as no enhancement of viral epitope exposure has been recorded for HIV-1NL4-3 (Sattentau, 1995; Grovit-Ferbas et al., 2000) or HIV-1MN in this study. Indeed, cells expressing gp160, treated with low concentrations of formalin (0.01%) alone, have been shown to increase binding of the 447d and 48d monoclonal antibodies (Sattentau, 1995). Therefore, the true effect of heat treatment on antigen exposure is unclear, as loss of antibody binding to gp120 has also been reported with heat inactivation at 56°C (for 2 hours) (Rossio et al., 1998).

Inactivation of HIV-1 using BPL reduced infectivity below the threshold of detection. Treatment with BPL produced some of the highest losses in viral RT activity, p24 and gp120 content among the inactivating agents tested. Race et al. (1995) used BPL to inactivate three strains of HIV-1, including HIV-1MN, by approximately $10^{20}$ TCID$_{50}$/ml. In this study by Race et al. (1995), similar reductions in p24 content post-inactivation were observed, but losses in gp120 were not as high. This may be due to combining cholesterol depletion with BPL inactivation as lower levels of gp120 loss were observed (20%) with BPL treatment alone. β-propiolactone has been reported to maintain protein antigenicity throughout the inactivation process (Logrippo et al., 1955; Race et al., 1995), and the data presented here confirm this.

Combination of BPL with other inactivation techniques, such as γ-irradiation, has been used to produce a gp120-depleted HIV-1 immunogen (Silvera et al., 2004). When tested by vaccination of rhesus macaques, this immunogen produced anti-p24 antibody responses to multiple Gag epitopes up to 6 weeks after the final immunisation, as well as inducing cell-mediated responses in 75% of animals (Silvera et al., 2004). However, the efficacy of a vaccine lacking the surface target immunogen, gp120, against subsequent challenge remains questionable. Evidence of this has been shown by the REMUNE clinical trial (2004), a phase III clinical trial testing a gp120-depleted whole-inactivated vaccine (“REMUNE”) for therapeutic use in infected individuals.
(Chantratita et al., 2004). Despite some increases in CD4+ T cell function, the study failed to demonstrate any increase in HIV-1 progression-free survival (Chantratita et al., 2004).

Aldrithriol-2 inactivates a number of different HIV-1 strains (MN and LAI) and SIV_{MNE} by up to $10^5 \text{TCID}_{50}/\text{ml}$ while allowing virions to retain both CD4 binding and fusion with target cells, indicating preservation of glycoprotein conformation (Rossio et al., 1998). Data presented here consolidates this, as complete inactivation of HIV-1_{MN} ($10^5$ log reduction in TCID$_{50}$/ml) was observed while retaining gp120 conformation, as assessed by b12 antibody binding. Indeed, antibody binding increased (with borderline significance, $p=0.08$) with combined cholesterol depletion and 1mM AT-2 treatment, due to the exposure of the b12 binding epitope. Assessment of RT activity post-inactivation was reduced by 60% using AT-2 alone or when combined with cholesterol depletion. As AT-2 specifically targets the NC and has been reported to be partly involved in reverse transcription by facilitating tRNA$^{\text{LYS}}$ primer annealing (Morellet et al., 2003), the reduction in RT activity observed may result from cross-linking of NC proteins (Rein et al., 1996). Although no significant reduction in viral p24 content was observed, virion-bound gp120 was reduced by 34% upon AT-2 treatment alone and by 79% when combined with cholesterol depletion which is a disadvantage of using AT-2 in the production of a whole-inactivated HIV-1 vaccine candidate.

$N$-Ethylmaleimide (NEM), targets the NC protein and inactivates HIV-1_{LAI} without affecting gp120 integrity (Morcock et al., 2005). Inactivation of HIV-1_{MN} using NEM substantially reduced viral infectivity by 5 logs, although residual infectivity was detectable. Morcock et al. (2005) demonstrated a reduction in TCID$_{50}$/ml of HIV-1_{LAI} of 6 logs while retaining fusion ability and, hence, glycoprotein conformation and function when using 1mM NEM as an inactivating agent. These higher reductions in infectivity compared to the data presented here could be due to the use of different viral strains. Alternatively, this degree of inactivation may have been achievable if higher titres of virus were used in this study. Viral RT activity was unaffected by NEM treatment, as previously reported (Morcock et al., 2005), however, gp120 and p24 protein content decreased by up to 20% for NEM treatment alone or when combined with cholesterol depletion. Importantly, the conformation of gp120 was preserved following inactivation.
It has previously been reported that AT-2 inactivation preserves viral-cell fusion shown by fusion-from-without in a syncytium induction assay (Rossio et al., 1998). This is the demonstration of fusion with cells without productive infection (Rossio et al., 1998). Therefore, this indicates functional glycoproteins that are able to facilitate fusion, however, inactivation of HIV-1 by disrupting the zinc fingers within the NC protein suspends viral replication at a stage after membrane fusion (Rossio et al., 1998). Although this was recorded for NEM, total inhibition of fusion was observed when HIV-1MN was treated with 1mM AT-2. Cholesterol depletion also led to a decrease in gp120 content of AT-2 inactivated virus which could be the cause of inhibition of cell fusion.

The nucleocapsid protein plays a pivotal role in reverse transcription (Introduction, sections 1.6.3 and 1.7.4), therefore, as AT-2 cross-links the NC protein, reduction in RT activity post-inactivation would most likely result from direct inhibition of reverse transcription. The advantage of compounds like NEM or AT-2 is high selectivity for HIV-1, for example they have no effect on human foamy virus (HFV), a spumaretrovirus, lacking zinc fingers in the nucleocapsid protein. Therefore, these compounds have been investigated as possible antiretroviral agents where non-toxic varieties can be found (for example, disulphide-substituted benzamide-4 (DIBA-4)) (Rein et al., 1996; Jenkins et al., 2005). Such compounds effectively suspend HIV-1 replication and provide little opportunity for viral escape, as the zinc finger NC amino acid sequence is highly conserved (Rice et al., 1995). Moreover, they have the advantage of retaining gp120 conformation (Rossio et al., 1998) and, thus, act in a way that allows viral fusion, with suspension of replication before viral reverse transcription and integration.

Advances in understanding the exact mechanism by which AT-2 and NEM inactivate HIV-1 have been made using compounds that do not target the NC. The compound 4-vinylpyridine (4-VP) does not react with zinc-chelated cysteine residues in NC, but can affect other viral proteins containing zinc finger structures (Morcock et al., 2008). Hence, 4-VP can show the importance of zinc finger cysteine residues in viral replication and the role of other cysteine-rich proteins in chemical inactivation. The use of 4-VP alone reduced viral infectivity 10-fold, but when combined with a membrane-
permeable chelator, targeting Zn$^{2+}$ residues in NC, infectivity was reduced to below the detectable range in a similar manner to AT-2 or NEM (Morcock et al., 2008). This shows that AT-2 and NEM target the NC protein directly and that the NC protein plays a pivotal role in viral replication. It is possible that AT-2 also interacts with IN, which possesses 6 zinc-chelated cysteine domains. The effect of AT-2 or NEM on RT activity may be explained by alterations in NC function as it acts as a chaperone during viral reverse transcription, hence, the reduction in RT activity with 1mM AT-2 inactivation observed in Figure 5.2.

Thus, the NC protein may be the ‘Achilles heel’ of retroviruses, as the cysteine residues involved in zinc chelation are highly conserved and present an attractive target for inactivating HIV-1. The recent discovery of taurine chloramine (T-NCI), a principal oxidative product of neutrophils, is important as it can inactivate HIV-1 effectively by removing NC zinc residues, in a similar way to AT-2 and NEM (Dudani et al., 2008). In mouse experiments, T-NCI-inactivated MMuLV vaccination protected all animals against challenge 38 weeks after the first immunisation (Dudani et al., 2008). The advantage of T-NCI is that it is a natural compound found within the body and would not require purification away from the virus after inactivation. This is unlike toxic compounds, such as AT-2 or NEM, which require post-inactivation purification, and may lead to changes in gp160 conformation as a result. Despite this, the development of compounds, like AT-2 or NEM, that can inactivate HIV-1 whilst preserving glycoprotein antigenicity could prove useful in the future for ex vivo quantification of T cell immune responses in infected patients or to test immune responses resulting from vaccination against HIV-1 (Rutebemberwa et al., 2007).

It is important to repeat the work in this chapter to ensure that the results can be statistically assessed. After correcting for multiple statistical comparisons against untreated virus by Bonferroni’s correction, no significant differences were observed by non-parametric tests for this data. Therefore, it is important to repeat all experiments at least twice to gain a robust picture of the effect of cholesterol depletion and inactivation of HIV-1$_{MN}$.

There are a number of parameters that should be explored to assess HIV-1 chemical inactivation, including the amount of time the virus is incubated with the inactivating
agent, the pH of the solution in which inactivation occurs, as well as the range of concentrations of inactivating agent. Similarly, inactivation of virus could be done before cholesterol-depletion or vice versa. Exploring many new and already available inactivating agents along with inactivation parameters discussed is needed to assess the most effective way of ablatting HIV-1 infectivity before this approach could be considered for vaccine purposes.

In this chapter we investigated the potential for reducing viral infectivity with different inactivating agents. All reduced HIV-1 infectivity to below detectable levels. Although NEM ablated viral infectivity, assessed by PBMC titration, there was evidence of viral fusion and, therefore, a total reduction in infectivity is questionable. Inactivation of virus using 0.8% formalin is also an unsuitable treatment for producing a whole-inactivated HIV-1 vaccine because despite potency in the reduction in viral infectivity, there was considerable loss in gp120 conformation.

Although glycoprotein content was lost upon viral inactivation using BPL or AT-2, with AT-2 creating the largest loss in gp120, antibody binding increased when viral samples were normalised to post-inactivation virus-associated gp120. After normalisation of antibody binding to gp120, a 6- and 5-fold increase in antibody binding was observed for AT-2 and BPL, respectively. The key parameter in assessing inactivating agents is maintenance of the gp120 conformation combined with the effective reduction of viral infectivity. The compound selected as the inactivating agent in producing a cholesterol-depleted whole-inactivated HIV-1MN vaccine immunogen for further in vivo investigations was AT-2, as it retained gp120 conformation whilst inactivating virus. Large quantities of AT-2 inactivated virus could also be acquired through the NIH AIDS reagents program, giving a source of HIV-1MN that was certified as inactivated.
Chapter 6:

Immunogenicity of cholesterol-depleted HIV-1 \textit{in vivo}.
6.1 Introduction

More than 25 years after the isolation of HIV-1 as the aetiological agent of AIDS, a vaccine remains a distant prospect. The concept of a whole-inactivated HIV-1 immunogen is now being revisited (Race et al., 1995; Race et al., 1995; Poon et al., 2005; Poon et al., 2005), despite being unacceptable more than a decade ago (Niedrig et al., 1993). Abandonment of whole-inactivated HIV-1 immunogens has mainly been due to concern over infection resulting from vaccination by virus that is insufficiently inactivated.

Investigation of whole-inactivated HIV-1 as an immunogen remains a worthwhile approach, given inactivated biological immunogens are the basis of many commercial vaccines, including the current Hepatitis A (Merck or GlaxoSmithKline), polio (Aventis Pasteur) and Rabies (Sanofi pasteur) vaccines (Appendix 1).

One advantage of a whole-inactivated vaccine approach is the likelihood of stimulating both cell-mediated and humoral immunity by exposing the immune system to a complex mixture of viral antigens (Kitabwalla et al., 2005; Kitabwalla et al., 2008). Furthermore, it would allow the presentation of native trimeric gp160 structures within the context of the viral envelope, the oligomerisation of envelope being apparently essential for eliciting neutralising antibodies (Roben et al., 1994; Sattentau et al., 1999).

Experiments with whole-inactivated retroviral immunogens, such as FIV and EIAV have confirmed the potential of such approaches in the prevention of retroviral infection (Marx et al., 1986; Desrosiers et al., 1989; Murphey-Corb et al., 1989; Sutjipto et al., 1990; Issel et al., 1992; Stahl-Hennig et al., 1992; de Vries et al., 1994; Hosie et al., 1995; Stott et al., 1998; Hosie et al., 2000; Dunham, 2006; Dunham et al., 2006). A study involving vaccination of ponies with inactivated EIAV protected animals against challenge with homologous virus and this protection correlates with both cell-mediated and humoral immunity (Issel et al., 1992). Similar results have been observed in cats given an inactivated FIV vaccine (Hosie et al., 1995; Hosie et al., 2000). Section 1.10.2 of the introduction discusses inactivated retroviral vaccines in more detail.
Whole-inactivated HIV-1 immunogens have also been shown to induce neutralising antibody responses to homologous and heterologous HIV-1 strains in animal studies (Race et al., 1995; Race et al., 1995; Poon et al., 2005; Poon et al., 2005). For example, Poon et al., (2005) demonstrated that serum taken from mice vaccinated with whole-inactivated HIV-1\textsubscript{SX} could achieve 50% neutralisation of the homologous strain at a 1 in 50 dilution. Similarly, 50% neutralisation was also observed in 3 of the 5 mice when tested against a range of heterologous strains (clades A, C, E, B and group O isolates) at a 1 in 20 dilution. Thus, evoking immune responses to HIV-1 by exploiting the development of whole-inactivated HIV-1 immunogens is an achievable goal, especially if neutralising antibody responses that are often weak or absent after immunisation can be induced (Jiang et al., 2005). In section 1.10.3 of the introduction inactivated HIV-1 vaccines are discussed in more detail.

The data set out in Chapters 3 and 4 show that cholesterol depletion enhances HIV-1 antibody binding and neutralisation by MAbs, sCD4 and patient antisera, while having little effect on virus gp120 content. Work in chapter 5 demonstrated the rational for selection of AT-2 as the inactivating agent for investigating the immunogenicity of cholesterol-depleted, whole-inactivated HIV-1\textsubscript{MN} in vivo. This compound was chosen because of it’s ability to inactivate HIV-1 while conserving the conformational neutralising epitopes of gp160.

In this chapter, we evaluate the in vivo immunogenicity of whole-inactivated, cholesterol-depleted HIV-1 in mice. Antibody responses raised were compared to mice vaccinated with native inactivated virus. This preliminary experiment was carried out in a murine model in the first instance to determine the degree of immunogenicity of cholesterol-depleted whole-inactivated HIV-1, before exploring animal models to analyse the potential for generating neutralising antibodies.

Subcutaneous immunisations, tails bleeds and mouse sacrifice was carried out by Dr Simon Jeffs or trained staff at the animal housing facility. All other work in this Chapter was carried out by the author.
6.2 Results

Immunogen preparation and mouse immunisation

AT-2 inactivated HIV-1\textsubscript{MN} was subjected to 1mM MBCD treatment, purified by ultracentrifugation and tested for viral envelope cholesterol content (Materials and Methods, sections 2.8 and 2.9, respectively). All cholesterol-depleted virus had a 50% reduction in cholesterol compared to untreated controls (data not shown).

Table 6.1 details the vaccination groups, with each group containing 5 BALB/c mice. The positive control group was AT-2 inactivated HIV-1\textsubscript{MN} (Group A) and was used as a comparison for antibody responses raised against cholesterol-depleted AT-2 inactivated virus (Group B). Uninfected cell supernatant, purified in the same way as HIV-1\textsubscript{MN}, was treated with or without 1mM MBCD (Group C and D, respectively) and used to identify non-specific antibody responses to vaccination. As the viral preparations were unlikely to induce immune responses in the absence of adjuvant, viral and control immunogens were mixed with Titermax Gold (Invitrogen) in a 1:1 (v/v) ratio. All the mice in each group received identical immunogen preparations. The preparations were dispensed into syringes in 100µl volumes ready for immunisation.

Before \textit{in vivo} work commenced, total abolition of HIV-1\textsubscript{MN} infectivity after AT-2 treatment was verified by prolonged PMBC culture and testing for RT activity at regular intervals over 4 weeks (Figure 6.1). Reverse transcriptase activity dropped to undetectable levels within one week of culture and remained undetectable thereafter.

To confirm these results, viral inactivation was assessed using the C8166 cell infectivity assay (Materials and Method, section 2.11). Syncytia were initially observed after 3 days of addition of virus to the cells, but passage of both C8166 cells and supernatant from initial viral cultures onto fresh C8166 cells resulted in no syncytia being induced (data not shown). Passaging of the cells and supernatant in this way indicated that the syncytia observed after 3 days resulted from fusion from without and not productive infection. No further syncytia were observed over 28 days, confirming complete inactivation of the virus.
Table 6.1. Specification of immunogens.

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Group</th>
<th>AT-2</th>
<th>MBCD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inactivated native virus</td>
<td>A</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Inactivated, cholesterol-depleted virus</td>
<td>B</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Inactivated supernatant</td>
<td>C</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Inactivated, cholesterol-depleted supernatant</td>
<td>D</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

Table 6.1 indicates the immunogens administered subcutaneously in a total volume of 100µl per mouse. The immunogen was given to mice in groups of 5 with each group being kept in individual cages. Concentrated HIV-1<sub>MN</sub>, grown in SupT1 cells and inactivated with AT-2, was obtained through the NIH AIDS Reagent Program. Cholesterol-depleted virus was subjected to 1mM MBCD treatment before purification by ultracentrifugation (Materials and Methods, section 2.8). Uninfected SupT1 cell supernatant (microvesicle control also provided by NIH), was treated with or without 1mM MBCD in the same way as viral samples and used as the negative controls. The vaccination schedule is detailed in section 2.29.
Figure 6.1. Infectivity of AT-2 inactivated HIV-1.

Figure 6.1. Infectivity of AT-2 inactivated HIV-1\textsubscript{MN} (from NIH) was assayed to ensure virus was fully inactivated. Virus was diluted 1/10 from concentrated stock (1mg/ml total protein) in PBS and incubated with $1 \times 10^5$ PBMCs in a 96-well tissue culture plate. PBMCs had been pre-stimulated for 3 days with PHA. Culture supernatant was periodically harvested over 28 days (x-axis) and tested for RT activity (y-axis) by qRT-PCR (section 2.12). Error bars represent standard deviation of the mean from a single experiment carried out in triplicate.
The four groups of mice (labelled group A-D, Table 6.1) were given a primary subcutaneous (prime) immunisation on Day 0, followed by two further subcutaneous (boost) immunisations on Days 21 and 44, respectively (Figure 6.2). The viral immunogens contained the gp120 and p24 concentrations indicated in Table 6.2 and each immunisation dose was quantified by p24 content and matched to control supernatant preparations by total protein content.

Table 6.2 shows that secondary treatment of the boost preparation resulted in loss of gp120, but not p24 (0.2µg per mouse compared to 0.6µg and 0.8µg for the prime and second boost preparations, respectively). This may be due to the shedding of gp120 during extended virus manipulation, resulting from a second 1mM MBCD treatment in order to obtain the required level of cholesterol depletion (50% reduction in viral envelope cholesterol) that was not achieved during the first MBCD treatment.

Figure 6.3 shows a Western blot of all immunogen preparations used in this study. The results suggest that cholesterol-depleted HIV-1MN virions retain antigenicity as evidenced by the strong binding of polyclonal and monoclonal antibodies raised against gp120, gp41 and p24 (Figure 6.3A) or by using pooled HIV-1 patient antiserum (Figure 6.3B) showed no changes to protein composition of the virus. Control uninfected cell supernatant preparations treated with or without 1mM MBCD indicated viral antigens were not present.
Figure 6.2. Mouse vaccination and test bleed schedule.

<table>
<thead>
<tr>
<th>Day 0</th>
<th>+14</th>
<th>+21</th>
<th>+36</th>
<th>+44</th>
<th>+55</th>
<th>+75</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-bleed</td>
<td>Prime vaccination</td>
<td>Test bleed 1</td>
<td>1&lt;sup&gt;st&lt;/sup&gt; Boost</td>
<td>Test bleed 2</td>
<td>2&lt;sup&gt;nd&lt;/sup&gt; Boost</td>
<td>Final Test bleed</td>
</tr>
</tbody>
</table>

Figure 6.2. Vaccination schedule to assay <i>in vivo</i> immunogenicity of cholesterol-depleted, whole inactivated HIV-1<sub>MN</sub>. Four groups of 5 mice (refer to Table 6.1) were given 3 days to acclimatise to laboratory conditions before a pre-bleed was taken from the tail vein to evaluate baseline antibody responses. The prime vaccination was given on Day 0 by subcutaneous injection, followed by two boost inoculations on Day 21 and 44, respectively. Test bleeds were taken from the tail vein approximately 2 weeks after each vaccination and the serum fractionated to analyse antibody titres. Animals were sacrificed by cervical dislocation on Day 75 and the spleens removed to isolate white blood cells.
Table 6.2. Envelope glycoprotein and p24 content of HIV-1 immunogens.

<table>
<thead>
<tr>
<th>Immunisation</th>
<th>gp120 (µg per animal)</th>
<th>p24 (µg per animal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prime (Day 0)</td>
<td>0.6</td>
<td>1.7</td>
</tr>
<tr>
<td>Boost 1 (Day 21)</td>
<td>0.2</td>
<td>1.8</td>
</tr>
<tr>
<td>Boost 2 (Day 44)</td>
<td>0.8</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 6.2 shows the HIV-1 protein content of each immunisation. Quantity of gp120 and p24 in each prime or boost immunisation. Immunogens were prepared as described in the legend for Table 6.1. Protein content was assayed by ELISA (Materials and Methods, sections 2.13 and 2.14). Control preparations were matched to viral samples by total protein content.
Figure 6.3. Western blot of whole-inactivated HIV-1.

Figure 6.2 shows a Western blot of inactivated HIV-1MN. Virus was prepared as described (Table 6.1) and 10μl of each immunogen loaded onto SDS-PAGE 4-20% Tris-glycine gradient gels for protein separation and transferred electrophoretically onto nitrocellulose membranes (Materials and Methods, section 2.15). Proteins were detected using A. gp120, gp41 and p24-specific monoclonal or polyclonal antibodies (section 2.15) and B. pooled HIV-1 positive antisera. The positive control consisted of HIV-1MN virus (5μg of total protein) and gp120, gp41 and p24 detected with the antibodies used in A.
Analysis of antibody responses to HIV-1 proteins

Blood was taken from mice two weeks after each vaccination and the sera analysed for the presence of antibodies to HIV-1 gp120, gp41 and p24 (Figure 6.2). Tail vein blood samples were allowed to clot at room temperature for 30 minutes and the cellular fraction pelleted by centrifugation (Materials and Methods, section 2.31). Sera were carefully removed, aliquoted and stored at -20°C before analysis of antibody titres by antibody capture ELISA. The antigens used in this assay were: recombinant HIV-1$_{111B}$ gp120 (IIIB gp120), homologous virus (AT-2 inactivated HIV-1$_{MN}$), clade C recombinant gp140 (ZN96) and p24 (Materials and Methods, section 2.31).

Antibody titres were quantified after each test bleed and were found to be greatest (> 1/1000 against IIIB gp120) after the second boost vaccination (on Day 44). Final blood samples were taken on Day 55 (after the second boost) and all subsequent serum analyses were carried out using sera taken at this time point. Pooled pre-bleed serum, taken 3 days prior to the priming immunisation (Day 0), served as the negative control to determine the cut-off value of the ELISA.

Figure 6.3 shows titration of mouse sera in an antibody capture ELISA using the antigen IIIB gp120. Sera from mice in each group were pooled and diluted in PBS containing 1% pig serum, as indicated on the x-axis. Group A and B absorbance values were corrected for non-specific background responses by subtracting the absorbance from the corresponding control Groups C and D, respectively. Across the full dilution series, two-fold increases in antibody titre were observed with cholesterol-depleted, inactivated virus (group B) compared to the untreated control (group A, $p<0.001$). Higher antibody titres against homologous virus (Figure 6.4) were also observed with cholesterol-depleted HIV-1$_{MN}$, although to a lesser extent (~1.5-fold). Tests using a recombinant clade C gp140 protein showed no antibody response to a clade C viral antigen (data not shown). Therefore, the antibody response was narrow in breadth and focused on homologous or closely related clade B viral antigens.

Antibodies raised against p24 (Figure 6.5) demonstrated no differences between any of the vaccination groups. Antibody titres of the negative control groups were, in fact,
Figure 6.3. Antibody responses to recombinant gp120 protein following immunisation.

Figure 6.3. Blood samples were taken from mice on Day 55 post-immunisation and plasma extracted from clotted whole blood by centrifugation. Sera from individual mice were pooled for each group and tested for antibodies raised against recombinant HIV-1 IIIB gp120 by ELISA (Materials and Methods, section 2.31). Antibody responses to native virus (blue) and cholesterol-depleted virus (red) were corrected for non-specific binding using the negative control groups C and D, respectively. Error bars represent standard deviation of the mean from a single experiment carried out in triplicate. Statistical tests on all dilutions showed significant differences ($p < 0.001$) between Groups A and B. Pre-bleed serum was used to determine the cut-off value for the assay.
Figure 6.4. Analysis of antibody responses to homologous virus post-immunisation.

Figure 6.4. Blood samples were taken as described in the legend for Figure 6.2 (Materials and Methods, section 2.31). The HIV-1 capture antigen used to test for antibody responses was identical to that used in preparing the vaccine (AT-2 inactivated HIV-1$_{MN}$). Groups A (blue) and B (red) were corrected for non-specific antibody binding using uninfected control groups C and D (untreated and 1mM MBCD treated uninfected cell supernatant), respectively. Error bars represent standard deviation of the mean from a single experiment carried out in triplicate. Antibody responses between the two groups were statistically significant ($p < 0.001$) at all dilutions. Pre-bleed serum was used to determine the cut-off value for the assay.
Figure 6.5. Analysis of antibody responses to p24 after immunisation.

Figure 6.5. Blood samples were taken on Day 55, as described in the legend for Figure 6.2 and sera pooled from individual mice (n=5) for each group. Antibodies raised against the HIV-1 p24 capsid protein were assayed by ELISA (Materials and Methods, section 2.31). Error bars represent standard deviation of the mean from a single experiment carried out in triplicate. Pre-bleed serum was used to determine cut-off values for the assay. Mouse groups are indicated in the key and correspond to those described in Table 6.1.
higher than the corresponding immunogen groups (e.g. group C absorbance was higher than group C by at least 0.3), indicating non-specific antibody responses to p24. Although the detection of antibodies against p24 after immunisation with the control preparations is unexpected, equal responses to p24 post-immunisation with test immunogens illustrated that cholesterol depletion did not affect the humoral response to p24.

**Analysis of individual murine antibody responses to HIV-1 proteins**

To investigate the degree of variation of immune responses between mice in each group after vaccination, the antibody titres of individual mice were assayed by antibody capture ELISAs against HIV-1IIIB gp120 or homologous virus using serum taken at Day 55. Figure 6.6 shows the antibody responses of mice against the IIIB gp120. Only one mouse from Groups A and B raised an antibody response recognising IIIB gp120 where the cut-off for non-specific responses was at an absorbance of 0.6 (measured at 450nm, Figure 6.6). This illustrates the inefficiency of eliciting antibodies to gp120 following immunisation. These two mice exhibited statistically-significant specific antibody responses, as no mice from the negative control groups responded similarly to vaccination. All other animals receiving test immunogens produced similar antibody responses to the control preparations, with absorbance values between 0.2 and 0.6. Although the median antibody titre was higher with vaccination using cholesterol-depleted HIV-1MN (Group B) compared to native virus (Group A), no significant difference was found.

All mice responded to the test immunogens and their humoral responses were greater than that observed in mice immunised with control preparations (except for one mouse in Group B), with absorbance values of greater than 1.2 at a 1/100 dilution (Figure 6.7A). However, there was no difference in median antibody titre against cholesterol-depleted compared to wildtype virus.

Figure 6.7B shows that when corrected for non-specific humoral responses by deducting corresponding control group absorbances (group C from A and D from B), cholesterol depletion led to an increase in median antibody titre, compared to the response to untreated virus. However, the difference observed between test groups A
Figure 6.6. Serological responses to recombinant HIV-1 gp120 post-vaccination.

Figure 6.6. Sera isolated at Day 55, as described in the legend for Figure 6.2, was tested for antibody responses to recombinant HIV-1\textsubscript{H1B} gp120. Individual mouse antibody responses were assayed by ELISA using a 1/100 serum dilution (Materials and Methods, section 2.31). The figure displays data from all groups with the cut-off value, determined using pooled prebleed sera, indicated with a red line.
Figure 6.7. Serological responses to homologous HIV-1 post-vaccination.

A

Experimental animal group

B

$p = 0.145$

Figure 6.7. Sera isolated at Day 55, as described in the legend for Figure 6.2, was tested for antibody responses to detergent disrupted AT-2 inactivated HIV-1MN (homologous virus). Individual mouse serum was tested by ELISA using a 1/100 serum dilution (Materials and Methods, section 2.31). A. displays all groups (A-D) with the cut-off value, determined using pooled pre-bleed sera, indicated by a red line. B. displays test groups corrected for background using pooled pre-bleed sera and responses of corresponding control preparations (groups C and D, respectively). The p value calculated using the Mann-Whitney test comparing groups A and B after correction for control preparation antibody responses in indicated.
and B after correction is a trend that was not significant. The amount of non-specific binding in this assay was high, as median titres from groups C and D gave absorbance readings of 1.1 to 0.9, respectively. This reflects the high non-specific antibody activity observed against the p24 protein (Figure 6.5).

The ability of mouse sera (1/100 dilution) to neutralise infectious HIV-1\textsubscript{MN} was analysed using the TZM assay (Materials and Methods, section 2.17) and showed no neutralising activity (data not shown).
6.3 Discussion

Initially, sera from individual mice were pooled and antibody reactivity to 4 HIV-1 antigens (recombinant IIIB gp120, homologous virus, p24 capsid protein and clade C ZM96 recombinant gp140) assessed. Two-fold increases in antibody titre against IIIB gp120 were observed with cholesterol-depleted HIV-1MN (group B) compared to untreated virus (group A). Similar increases were seen when sera were tested for immune responses to homologous virus.

All groups of mice (A-D) raised strong antibody responses to p24. This is not unexpected in the test groups (A and B) because immunogen preparations contained equal quantities of p24. Furthermore, cholesterol depletion should not alter the immune response to p24 (as this is not a membrane embedded protein). Despite there being no p24 present in the control preparations, as shown by Western blot (Figure 6.1), sera from groups (C and D) also exhibited antibodies against p24 indistinguishable from the test groups (A and B). These data indicated the presence of non-specific antibodies to the p24 protein in mice.

It has been observed that there is homology and antibody cross-reactivity between p24 and the structural proteins of other viruses, for example cytomegalovirus (CMV) or picornaviruses (Argos, 1989; Landini et al., 1991). This results from sequence and antigenic homology of the viral structural proteins that have similarities in their three-dimensional conformation (Argos, 1989). The Balb/c mice used in this study may have had underlying viral infections to which a memory humoral response was present. Immunisation with an HIV-1 based immunogen containing p24 with homology to other viruses could then have selected and expanded B cells leading to the production of high levels of antibodies cross-reactive with p24.

To explore the humoral immune response to the vaccine immunogens in more detail, the antibody titres from each individual mouse were measured. Although, the median antibody titre raised against the cholesterol-depleted HIV-1MN immunogen (group A) was higher than that against the untreated control (group B), this increase was not statistically significant. In fact, only one mouse in each of the test groups A and B raised antibody responses to recombinant IIIB gp120 that were significantly higher than
the responses of the control mice (p<0.001). Although HIV-1$_{IIIB}$ and HIV-1$_{MN}$ are both B clade viruses, this shows that producing robust cross-reactive antibody responses to gp120 within clades is difficult. This lack of cross-reactive antibody responses was further illustrated by the absence of antibody responses to a clade C glycoprotein, ZM96, a more distantly related HIV-1 strain. Refer to Section 1.3 for more details of the phylogenetics of HIV-1 isolates. The clade cross-reactivity of the serum produce after immunisation could be explored in more detail by testing a wider panel of HIV-1 isolates across all clades.

Investigations by Gorse et al. (1996) showed that immunisation of healthy, HIV-1 negative volunteers with recombinant HIV-1$_{IIIB}$ or HIV-1$_{MN}$ gp120 vaccines (produced in CHO cell lines) gave little cross-reactive antibodies between these two strains. Vaccination with recombinant HIV-1$_{MN}$ gp120 resulted in greater than two-fold reductions in antibody titre against HIV-1$_{IIIB}$ compared to homologous virus (Gorse et al., 1996). This reduction in antibody titre was also observed in the reverse situation, with a two-fold reduction in antibody titre against HIV-1$_{MN}$ compared to homologous virus after vaccination with HIV-1$_{IIIB}$ gp120 (Gorse et al., 1996).

The diversity across clades of HIV-1 can be illustrated by a difference in viral protein amino acid sequence of up to and in excess of 25%, depending on the protein analysed (McMichael et al., 2003). Even when comparing the diversity of viral proteins within a clade, the difference in clade B protein sequences can exceed 10%. Therefore, the production of antibodies able to react with multiple viral clades, or even multiple strains of HIV-1 within a clade, may be difficult (Gorse et al., 1996; Li et al., 2005). Developing a diverse humoral immune response post-immunisation can also depend on the type of antibodies provoked. For example, the CD4i antibodies in particular have effective cross-clade activity (Labrijn et al., 2003).

Elucidation of the epitopes responsible for eliciting humoral immunity in the two mice that did produce specific responses to IIIB gp120 may identify key epitopes that allow the generation of cross-reactive antibodies within clade B viral strains. This could be done by assaying the binding of mouse serum to overlapping peptides from IIIB gp120 by ELISA and could help to define the glycoprotein structures that easily produce antibody responses in vivo. It would also be crucial to optimise the protocol in order to
get more mice responding upon immunisation. This could be done by assessing a range of antigen concentration and immunisation schedules.

Individual mice from both viral immunogen groups elicited higher antibody titres against homologous virus compared with those raised against the heterologous antigen IIIB gp120. This was due to the detection of antibodies raised against the full complement of viral antigens and not gp120 alone. Control mice from groups C and D exhibited high absorbance values when assaying antibody titres against homologous virus. These high antibody titres most likely result from non-specific binding to homologous p24 viral protein in the capture assay.

Higher antibody titres against homologous virus were observed upon vaccination with cholesterol-depleted virus compared to the native control, but only when corrected for non-specific responses to control supernatant preparations. Despite this observed increase, the difference in humoral response between groups A and B was not significant, as illustrated by a Mann-Whitney \( p \) value of 0.145. This value is close to the 95\% confidence interval of the statistical test and suggests that a significant increase in antibody responses could be demonstrated if animal numbers per group were increased.

Cholesterol depletion of the control uninfected cell supernatant (Group D) elicited lower non-specific background responses to homologous virus antigens \( (p = 0.08) \) compared to uninfected cell supernatant alone (Group C). The MBCD treatment to remove cholesterol could have disrupted the small membrane fragments in the control preparations, so that after purification, the cellular protein content of these samples was reduced. Therefore, the difference in immune responses between Groups A and B may result from correction with corresponding control preparations and leads to questions regarding how best to control for whole-inactivated virus immunogens that contain cellular proteins within the viral envelope.

Immunity to xenoantigens within the viral envelope that convey protection against HIV-1 or SIV has been well documented (Murphey-Corb et al., 1989; Stott, 1991; de Vries et al., 1994). More detail on these experiments can be found in Section 1.10.2. These xenoantigens complicate results regarding humoral immune responses to vaccination.
with whole-inactivated viral immunogens. In order to control for xenoantigens present in viral preparations, it will be important to determine the host protein composition of both cholesterol-depleted and normal viral envelopes in order to account for this. This could be done by isolating viral envelopes using detergent extraction followed by the separation of proteins by electrophoresis and protein identification using mass spectrometry. However, this would be a laborious and time consuming task as large amounts of virus would be required to identify cellular proteins in this way.

An HIV-1 vaccine must elicit a neutralising antibody response in order to create sterilising immunity against infection. As increases in viral neutralisation with cholesterol depletion have been observed in vitro in Chapters 3 and 4, future work will be needed to assess the generation of neutralising antibody responses to cholesterol-depleted HIV-1\textsubscript{MN} in animal models. It is useful, however, to begin by deducing the immunogenicity of a vaccine preparation in mice, as this can illustrate increased in antibody titres with cholesterol depletion before beginning neutralising antibody animal investigations.

The production of neutralising antibodies could be better assessed in rabbits, guinea pigs, or macaques, as neutralising antibodies have been produced as a result of vaccination with HIV-1 immunogens in these models (Poon \textit{et al.}, 2005; Poon \textit{et al.}, 2005). For example, neutralising antibody titres to heterologous virus strains (clades A-D) in excess of 1/500 have been recorded after vaccination of macaques with inactivated HIV-1\textsubscript{SX} (produced using low dose formalin and thermal inactivation) (Poon \textit{et al.}, 2005).

Kitabwalla \textit{et al.} (2005, 2008) demonstrated an increased immune response, both cell-mediated and humoral, resulting from an AT-2-inactivated, cholesterol-depleted SIV vaccine in mice. Work here shows that these data may not be extrapolated to an AT-2-inactivated, cholesterol-depleted HIV-1 vaccine, due to several differences between work in this thesis and the SIV study. Firstly, Kitabwalla \textit{et al.} (2005, 2008) created cholesterol-depleted virus by solvent treatment, rather than MBCD treatment. Secondly, the SIV study incorporated incomplete Freund’s adjuvant (IFA) into the vaccination schedule, rather than Titermax Gold. Both of these factors could have led to differences in the immune responses gained.
Although mice were immunised with similar concentrations of prime antigen, Kitabwalla et al. (2005) boosted mice with ten-times less antigen than that used in this study. Compounded with this, the schedule of immunisation in the SIV study was not optimal for the development of humoral immune responses, as prime and boost vaccinations were given two weeks apart and mice sacrificed only four days after the boost injection (Kitabwalla et al., 2005). This brings into question whether sufficient time had been given to allow an optimal humoral immune response to develop.

Critically, Kitabwalla et al. (2005) did not control for xenoantigens in mice and, therefore, could not be assured that the cell-mediated and antibody responses recorded were virus-specific. Thus, overall comparisons between this study and the cholesterol-depleted SIV experiments are not straightforward.

The control of viraemia during HIV-1 infection is largely controlled by the CD8+ cytotoxic T cell (Borrow et al., 1994; Schmitz et al., 1999; Cloyd et al., 2001). These cells develop quickly after infection and are responsible for the destruction of HIV-infected cells throughout infection (Borrow et al., 1994; Schmitz et al., 1999; Cloyd et al., 2001). Therefore, mounting an effective cell-mediated immune response could be an important part of a protective immunity. The study by Kitabwala et al (2005) was aimed at enhancing cellular responses after vaccination with a lipid-depleted whole-inactivated SIV vaccine compared to live or AT-2 inactivated virus. Lipid depletion resulted in significant increases in IFNγ-producing CD4+ and CD8+ T cells responding to both Gag and Env antigens in mice, compared to the wildtype controls (Kitabwalla et al., 2005). For example, IFNγ-producing CD8+ T cells increased 2-fold upon cholesterol-depleted AT-2 inactivated virus compared to inactivated native virus (Kitabwalla et al., 2005).

Although attempts were made to analyse T cell responses and IFNγ secretion resulting from antigen stimulation post-vaccination, the results were inconclusive in this study. This is because primary T cells extracted from the spleens of vaccinated mice rapidly died in culture, even after 24 hours of culture with or without stimulating antigen. It would be important, however, to repeat these experiments in order to optimise the assay and prevent cell death. The rapid death of the primary T cells extracted from mouse
spleens could have been due to exposure to levels of antigen that were too high. Therefore, titrating the antigen used to stimulate T cell activation could lead to optimisation of the assay and enable in vitro primary T cell survival. In addition, the cell density per well could have been incompatible with cell survival.

The role of lipids in gp160 antigenicity is an area of intense exploration. Lipids have been shown to be important in the binding of the MAbs 4E10 and 2F5 to native virus and may initiate the binding of such antibodies before extraction of the partially lipid-buried epitopes (refer to section 3.3 for more details) (Haynes et al., 2005; Alam et al., 2007). The production of monoclonal antibodies, like 4E10 and 2F5, is being pursued in vaccine design, not only for their broad neutralising activity, but because they target residues of gp41 that are highly conserved as a result of their role in membrane fusion (HuarTE et al., 2008). This degree of conservation would lessen the likelihood of viral escape from a vaccine that produces this type of antibody.

In order to provoke antibodies like 4E10 and 2F5, immunogens incorporating lipid entities are being explored. Lipid A is a constituent of the lipid bilayer and was discovered as a potent adjuvant that resulted in the production of antibodies directed against liposomal lipids in 1979 (Schuster et al., 1979; Alving et al., 2006; Alving, 2008; Alving et al., 2008). One such antibody was directed against phosphatidylinositol 4-phosphate (PIP) that exhibited neutralising activity to HIV-1 infection (Brown et al., 2007). The anti-PIP antibody had phospholipid binding properties similar to the 4E10 antibody and, therefore, antibodies like anti-PIP could aid the production of HIV-1 neutralising antibodies in vivo (Brown et al., 2007).

Mice immunised with liposomes containing lipid A and either cholesterol with gp140 or galactosylceramide with MPER peptides induced antibodies able to bind homologous antigens (Beck et al., 2008). In a separate study, immunisation of mice with an HIV-1 MPER peptide covalently linked to lipids including cholesterol hemisuccinate (a cholesterol homolog) and cardiolipin produced high titres of antibodies to this peptide (Watson et al., 2009). However, these antibodies did not bind efficiently to gp140 showing that antibodies raised to peptides may not create antibodies capable of binding to native protein (Watson et al., 2009). Therefore, there is a structural aspect of these antigens that must be considered when eliciting antibodies to the MPER region.
Exploration of whole-inactivated HIV-1 vaccines has been limited and generally dismissed as a feasible vaccine strategy for HIV-1. Despite common use of whole-inactivated vaccines commercially, public fear over the risk of infection with a whole-inactivated HIV-1 vaccine may weaken public support, notwithstanding the possibility of producing a protective immune response.

The work in this thesis has demonstrated that cholesterol depletion of the virus envelope of primary (pseudotyped) and laboratory-adapted HIV-1 can increase antigenicity of viral glycoproteins without affecting viral gp120 levels. Furthermore, in vivo assessment of a whole-inactivated, cholesterol-depleted HIV-1 vaccine illustrated immunogenicity. Thus, cholesterol-depleted, whole-inactivated HIV-1 is an immunogen that has the potential to provide relevant humoral responses to HIV-1 and may prove useful in future HIV-1 vaccine strategies.
Chapter 7:

General Discussion
For over twenty-five years, since the discovery of HIV-1 as the etiological agent of AIDS, a quest has ensued to create an effective vaccine that would prevent infection. Despite all efforts, an HIV-1 vaccine is no closer to being produced than it was twenty-five years ago. However, a lot more about the obstacles that need to be overcome in the development of such a vaccine are now understood. The HIV-1 gp160 protein is highly developed to evade antibody attack by the immune system and, therefore, creating long lasting sterilising immunity to HIV-1 is difficult (Poignard et al., 1996; Wyatt et al., 1998; Sattentau et al., 1999).

The immune response during natural infection yields clues about the sort of immune response required for protection against HIV-1. However, the in vivo immune responses generated against HIV-1 infection are insufficient for the long-term control of virus replication or for viral clearance. The immune response that develops after HIV-1 infection is largely cell-mediated and this response can stabilise the level of infection and can be maintained in excess of 10 years (Borrow et al., 1994; Hadida et al., 1998; Schmitz et al., 1999; Lehner et al., 2000; Cloyd et al., 2001).

The function of a vaccine against any infectious disease is not to necessarily prevent an infection, but to limit the spread and to clear the microbe from the body by utilising a rapid secondary immune response derived from immunological memory (Iaccino et al., 2008). As HIV-1 is an integrating virus that establishes a latent, chronic infection, the development of an HIV-1 vaccine is more challenging. Vaccination should aim to create an immune response that is rapid and potent enough to clear all infected HIV-1 cells before viral mutation and/or latency is achieved (Iaccino et al., 2008). As this is unlikely to be achieved on induction of a secondary immune response which takes time to generate, this leads to the idea that only a continuous immune barrier can stop the initial infection (Iaccino et al., 2008). Therefore an ideal HIV-1 vaccine would prevent even 1 cell from becoming infected. To achieve this, an immune response which generates a potent continuous neutralising antibody titre that can neutralise virus before latent infection is required.

Due to the need to combat infection at the earliest stage, focus is moving to immunogens that stimulate mucosal immunity. Transmission across the genito-urinary and rectal mucosa can occur within 6 hours of exposure, with dissemination into local
lymphoid tissue within 24 hours (Haynes et al., 2008; Weiler et al., 2008). This rapid sequence of events establishes latent infection of cells in lymph tissue (Haynes et al., 2008; Weiler et al., 2008). Immunity derived from secondary immune responses must be activated within hours of exposure to virus for possible protective immunity. Hence, the option of inducing a mucosal immune response that includes neutralising secretory IgA and IgG antibodies, along with localised CTL-mediated immunity in the lymph tissue underlying the mucosa is an attractive one. To date, experimental attempts at producing an effective vaccine in animal models have, at best, protected from disease progression with stabilisation of viraemia post-challenge. Hence, the development of a mucosal vaccine may be the only way of creating sterilising immunity.

A limited number of broadly neutralising MAbs have been well characterised, illustrating the rarity of such molecules (Burton et al., 1991; Gorny et al., 1992; Muster et al., 1993; Thali et al., 1993; Buchacher et al., 1994). This is because HIV-1 gp160 glycoprotein is highly adapted to prevent antibody attack or to induce antibody production. The gp160 trimer possesses buried conserved regions that may contain neutralising epitopes and those amino acids sequences that are susceptible to neutralisation occur in regions only transiently exposed during receptor binding or membrane fusion (Sattentau et al., 1999; Zwick et al., 2001). Moreover, gp160 is heavily glycosylated, restricting antibody access to protein epitopes (Poignard et al., 1996). Furthermore, in order to elicit neutralising antibody responses by immunisation, native trimeric glycoproteins are essential and may need to be presented within a membrane (Luke et al., 1996; Cho et al., 2001). Despite overcoming these obstacles to produce a neutralising antibody response, the high mutation rate of HIV-1 (Roberts et al., 1988; Mansky et al., 1995) could create viral quasispecies able to escape immune regulation, rendering this initial neutralising antibody response useless.

Along with the question of what constitutes a sterilising immune response to HIV-1 and the difficulty in producing neutralising antibodies, significant worldwide HIV-1 strain diversity creates a further obstacle for producing a single universal HIV-1 vaccine. Given the production of a vaccine able to produce effective humoral immunity based on one immunogen, it is difficult to determine whether one type of response would protect an individual against all circulating HIV-1 clades, or even different strains within a single clade. The difficulty, therefore, lies in deciding whether to tailor HIV-1 vaccines...
to different geographical regions, targeting the most prevalent clades, or to design a ‘one size fits all’ vaccine able to create an immune response effective for inter- and intra-clade diversity. The problem with a regionally tailored vaccine is that the prevalent clade may simply be replaced with another clade or clades. Whereas designing a universal vaccine will be more challenging with respect to creating an immunogen able to protect against diverse HIV-1 clades.

Given any potential immunogen, the challenge is to test its efficacy in vivo. Animal models have limited value in the assessment of HIV vaccines, as HIV-1 is restricted to infecting humans and chimpanzees and only causes disease in humans (Puls et al., 2006). The use of SIV as an HIV-1 model is common, but of limited relevance, because it is non-pathogenic in the natural host and only causes disease in closely related primate species. The development of HIV-SIV chimeras (SHIV’s) has been useful in providing better models for HIV-1 pathogenesis (Stott et al., 1998), but the lack of a way to directly assess HIV-1 immunogens remains a disadvantage.

Although it has long been thought that SIV does not cause disease in the natural host, it has recently been described that chimpanzees living naturally in the wild do exhibit symptoms typically associated with HIV-1 infection in humans (Keele et al., 2009). In a recent study of wild chimpanzees in Tanzania, those infected with SIV<sub>cpz</sub> had a 10- to 16-fold increase in early death compared to uninfected chimpanzees (Keele et al., 2009). Infection with SIV<sub>cpz</sub> was also associated with CD4<sup>+</sup> T cell depletion, high viral load and histopathological findings associated with end-stage AIDS on postmortem (Keele et al., 2009).

One other tool that could be used to aid HIV-1 immunogen design is the use of a potent adjuvant to increase immune responses by immunisation. There is extensive investigation into this area of vaccinology and it is one that crosses research boundaries. For example, proteins secreted by parasites have been widely studied for their effect on the immune response during parasitic infection. These proteins have been used to explore novel adjuvants for use in HIV-1 vaccines (MacDonald et al., 2005). A parasite protein from a filarial nematode, Onchocerca volvulus activation associated protein-1 (rOv-ASP-1), has been used as an adjuvant in conjunction with immunisation of mice with Ovalbumin (MacDonald et al., 2005). When compared against a control group
immunised with Ovalbumin constituted with Alum adjuvant, rOv-ASP-1 produced a greater antibody and cell-mediated immune response post-immunisation (MacDonald et al., 2005). As well as creating antibody responses after immunisation with a gp120-CD4 chimeric protein mixed with the rOv-ASP-1 parasite protein adjuvant, this approach generated cell-mediated immunity that included the augmentation of Th1 and Th2 cellular immune responses, the latter being instrumental in the potential of the anti-viral response (MacDonald et al., 2005).

Investigation of different inoculation routes has shed light on the potential advantages of targeting an HIV-1 vaccine to specific tissues. Several studies involving formalin-inactivated SIV, encapsidated into poly(DL-lactide-co-glycolide) (DL-PLG) microspheres, led to interesting results in mouse models in which increased antibody titres were observed compared to formalin-inactivated SIV alone (Marx et al., 1993). Extension of this work to investigate different inoculation routes indicated that an intramuscular prime followed by a mucosal boost achieved protection from challenge in 5 of 6 macaques (Marx et al., 1993). However, intramuscular prime and boost immunisation, intramuscular prime plus oral/intra-tracheal boost or vaccinia prime (antigens) with intra-tracheal boost produced no protection against challenge, despite eliciting IgG and IgA antibodies in serum, as well as in the vaginal and tracheal tracts (Ishizaka et al., 1999; Israel et al., 1999).

More invasive inoculation techniques used by Lü et al (1998) targeted iliac lymph nodes for vaccination, a process by which immunogen is deposited specifically at these iliac lymph node sites. This technique failed to protect animals upon challenge, despite the development of both serum and vaginal IgG and IgA (Lu et al., 1998). This study involving a different immunisation site shows that it may be important to investigate the mode of vaccine delivery to gain the most effective immune response against infection.

The data presented in this thesis have shown that depleting the HIV-1 envelope of cholesterol can increase the epitope exposure of gp160. Increases in interaction of HIV-1MN virus with MAbs and viral neutralisation were observed at multiple epitopes at locations close to and distant from the membrane. Antibody binding (indirectly assessed) and virus neutralisation are increased when cholesterol is removed from the viral envelope and this could be due to increased flexibility of the glycoproteins within
the bilayer, or result from subtle changes to the conformation of the glycoproteins embedded in the lipid envelope.

A panel of HIV-1 primary isolates was screened in order to identify those with the highest levels of associated gp120. This was done to select an isolate with sufficiently high gp120 for cholesterol depletion studies on primary HIV-1 isolates. The titre of virus that could be achieved in primary culture (defined as no more than 3 passages in vitro) was insufficient for the study of cholesterol-depleted primary HIV-1 and, therefore, it was decided that a pseudotyped isolate, with titres similar to those of a laboratory-adapted isolates, would be used as an alternative. Cholesterol depletion of a pseudotyped primary B-clade isolate effected changes in epitope exposure to a lesser extent than that seen with HIV-1-MN, but this could have been due to the single quasispecies nature of the pseudotyped primary HIV-1 used. Incorporation of greater larger viral diversity, by using a variety of envelope sequences, may increase antigenicity with cholesterol depletion of primary HIV-1.

Once it had been established that cholesterol depletion can increase the exposure of viral epitopes, investigations were undertaken to create a cholesterol-depleted, whole-inactivated HIV-1 immunogen to in vivo immunogenicity. To this end, a number of previously explored methods of inactivation were considered to determine the best way in which to inactivate cholesterol-depleted HIV-1-MN while preserving the native gp160 conformation. These experiments were carried out with a laboratory-adapted HIV-1 strain, not a primary isolate, which may represent a more effective immunogen in terms of mimicking circulating isolates. Cholesterol-depleted, whole-inactivated HIV-1-MN was prepared as an immunogen to determine the effect of cholesterol removal on the immunogenicity of virus in vivo. However, due to the amount of glycoprotein required for vaccination (at least 1µg per animal), it was not feasible to propagate sufficient primary virus for these in vivo studies. Therefore, the laboratory-adapted strain, HIV-1-MN, was used in a pilot proof of principle experiment to demonstrate the changes in immunogenicity with cholesterol depletion of a whole-inactivated HIV-1 immunogen.

The reagent AT-2 was chosen for inactivation of cholesterol-depleted, whole-inactivated HIV-1-MN on the basis of its ability to effectively inactivate the virus, while preserving the protein content of the virus and maintaining the antigenicity of gp160.
For the *in vivo* experiments, mice were subcutaneously immunised with approximately 2µg of p24 for a total of 3 immunisations and blood samples taken to assess serum antibody titres.

Pooled serum from mice illustrated immunogenicity of both native and cholesterol-depleted virus. In antibody capture ELISA’s using both HIV-1<sub>IIIb</sub> recombinant gp120 and homologous virus as antigens, the latter exhibiting a 2-fold increase in antibody titre compared to the native virus control. However, when individual mouse responses were tested against the same immunogens, although a slight increase in median antibody titre was observed with cholesterol-depleted virus compared to the native virus immunogen, no significant differences in antibody titres were observed between these groups.

These data are preliminary. Never-the-less, they give an indication of the potential for cholesterol depletion as a tool to increase exposure of viral epitopes and enhance the immunogenicity of whole-inactivated virus. This is especially true with respect to the epitopes found in the MPER region of gp41, as it was in this area that the largest increases in antibody interaction with virus and neutralisation of HIV-1 infectivity were observed after cholesterol depletion by using the Mabs 4E10 and 2F5. It would seem, therefore, that the environment of the viral envelope protein plays a key role in their antigenicity.

Antigenicity and immunogenicity can be increased by changes in cholesterol that increase the transition temperature, reduce the microviscosity and, hence, increase the ‘rigidity’ of the liposomal membranes (Borochov *et al.*, 1976; Shinitzky *et al.*, 1979). Removal of cholesterol facilitates greater accessibility to antigens, enhanced binding to immune cells followed by endocytosis and results in an enhanced primary immune response (Yasuda *et al.*, 1977; Batenjany *et al.*, 2001).

Changing cholesterol quantities has been used in the past to alter the properties of cancer vaccine immunogens. Immunisation of mice with irradiated B and T lymphoma cells, possessing elevated membrane cholesterol, increased animal survival upon challenge (Shinitzky *et al.*, 1979). This effect was associated with vertical
displacement of membrane antigens leading to a more potent immune response (Borochov et al., 1976; Shinitzky et al., 1979).

The safety issues surrounding an inactivated HIV-1 vaccine, dictate the need to study both liposomal structures and replication-deficient HIV-1 containing different cholesterol compositions. This might pin-point a membrane lipid composition that could yield optimal exposure of membrane-bound antigens. Liposomal structures also represent whole viral alternatives that could lead to a clarified role of the lipid environment in the presentation of antigenic proteins and define the structural changes involved.

The naturally raised cholesterol levels observed in the HIV-1 envelope, which result from viral budding from cell membrane lipid raft regions (Aloia et al., 1993; Nguyen et al., 2000; Munro, 2003; Brugger et al., 2006), may add to the masking of gp160 epitopes. Thus, removal of cholesterol from the viral envelope, enabled the exposure of these epitopes. Cholesterol been shown to be essential for viral fusion, whereby increased cholesterol in the viral envelope aids lipid mixing (Manes et al., 2000; Guyader et al., 2002). In addition, viral assembly at cholesterol rich lipid raft regions of the cell membrane allows the concentration of viral proteins, as well as attracting Env and other HIV-1 proteins into progeny virions (Vincent et al., 2002; Ono et al., 2007). The HIV-1 virus could have evolved to incorporate increased amounts of cholesterol in its envelope in order to assist in multiple parts of its replication pathway. Increased cholesterol in the viral envelope could allow evasion of immune attack by contributing to the masked nature of the gp160 epitopes (Wyatt et al., 1998). The virus with increased envelope cholesterol could have been selected for by the survival advantages associated with the role of cholesterol in viral replication and evasion of immune attack.

So how do we move forward from 25 years of HIV-1 vaccine research to develop an effective HIV-1 immunogen? There is a case for exploiting both classical approaches to vaccine design, as well as investigating novel and unique approaches to vaccinology. Firstly, how do we mimic the structures that can produce broadly neutralising antibodies to HIV-1? One difficulty is that there is a structural aspect to epitopes that yield such antibodies, for example the 4E10 epitope (Frey et al., 2008; Veiga et al., 2009). The
4E10 epitope is only transiently exposed during fusion and also encompasses membrane lipid interactions which are self antigens and are unlikely to be induced (Frey et al., 2008; Veiga et al., 2009). The design of non-HIV-1 structural scaffolds for presenting conserved neutralising epitopes is one objective of The Collaboration of AIDS Vaccine Discovery team (www.cavd.org). This may produce unique protein scaffolds that can present neutralising epitopes to the immune system in order to induce strong neutralising antibody responses in vivo.

It is theoretically possible that neutralising antibodies could also be produced using anti-idiotypic antibodies. A study by Burioni et al. (2008) showed that purified IgG from mice immunised with serum from a LTNP yielded hybridomas that produced antibodies recognising the idiotypic (defined as the portion of an immunoglobulin molecule that confers the molecule's unique character, most often including its antigen-binding site) of the patient serum (in particular a b12 like antibody). Fab fragments of these anti-idiotypic antibodies were then successfully used to induce a neutralising anti-gp120 response after immunisation of rabbits. This neutralising antibody response could neutralise similar HIV-1 isolates to the patient strain (Burioni et al., 2008). Extension of this work to increase the strength and breadth of the neutralising antibody response generated, broadly acting neutralising antibodies could be provoked by anti-idiotypic Fab fragments (Burioni et al., 2008), although early studies using this approach were not rewarding.

Novel work involving the expression of intracellular antibodies (intrabodies) has given insight into the development of promising strategies for preventing or controlling HIV-1 infection in the future (Marasco, 1997; Rondon et al., 1997). An intrabody is an antibody or antibody fragment that can be expressed and interact with antigen intracellular (Marasco, 1997; Rondon et al., 1997). Work by Chen et al. (1994) showed that CD4+ T cell expression of Fab fragments of a neutralising antibody targeting the CD4 binding site, F105, could bind and neutralise both intracellular virus and cell-free virus on secretion of these Fab fragments. This effectively rendered the CD4+ T cells expressing these antibody fragments resistant to HIV-1 infection and able to protect neighbouring cells by secretion of neutralising Fab fragments (Chen et al., 1994).
Development of the intrabody idea has led to the targeting of HIV-1 proteins other than the glycoproteins, such as Tat or RT. This may lead to an approach that can minimise viral escape by targeting proteins that are more conserved than gp160. Mhashilkar et al. (1995) produced an antibody construct that could produce anti-Tat intracellular antibodies. This construct was also modified to allow nuclear localisation of the intrabodies in order to block Tat transactivation of the HIV-1 LTR and, hence, ablate viral replication (Mhashilkar et al., 1997). As a result, transformed lymphocytes expressing anti-Tat intrabodies were resistant to HIV-1 infection (Mhashilkar et al., 1997).

Furthermore, investigation of the expression of intrabodies directed against RT has also been shown to render CD4+ T cells resistant to HIV-1 infection (Maciejewski et al., 1995; Mhashilkar et al., 1997). With advancements in gene therapy, the production of intrabodies against HIV-1 proteins like RT and IN could be a potent mechanism of preventing HIV-1 infection (Maciejewski et al., 1995; Mhashilkar et al., 1997). If the targeting of lymphohaemopoietic stem cells or mature lymphocytes for gene therapy with anti-HIV-1 intrabodies can be achieved, gene transfer intracellular immunisation may prove to be a feasible HIV-1 prevention technique (Marasco, 1997; Rondon et al., 1997).

In conclusion, this study has examined the use of cholesterol depletion as a mechanism of increasing the antigenicity and immunogenicity of the whole-inactivated HIV-1 vaccine approach. Despite the extensive work in developing HIV-1 immunogens, an effective HIV-1 vaccine remains elusive. However, with multi-national research consortium initiatives and the continuation of pushing the boundaries of vaccine research to encompass novel ideas, the goal of producing an effective HIV-1 vaccine able to prevent infection arguably remains achievable.
Chapter 8:

References


Comparison of vaccine strategies using recombinant env-gag-pol MVA with or without an oligomeric Env protein boost in the SHIV rhesus macaque model. Virology 294:270-81.


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UNAIDS. 2007. UNAIDS/WHO AIDS.

UNAIDS. 2008. UNAIDS/WHO AIDS.


Chapter 9:

Appendices
Appendix 1: Current vaccine strategies

<table>
<thead>
<tr>
<th>Virus</th>
<th>Innoculation route</th>
<th>Inactivated</th>
<th>Attenuated</th>
<th>Recombinant protein/subunit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatitis A</td>
<td>Im</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatitis B</td>
<td>Im/Sc</td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Human pappiloma virus (types 16 and 18)</td>
<td>Im</td>
<td></td>
<td></td>
<td>VLP</td>
</tr>
<tr>
<td>Influenza</td>
<td>Im</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Japanese B encephalitis</td>
<td>Sc</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Measles, mumps, rubella (MMR)</td>
<td>Im</td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Polio</td>
<td>Im</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polio</td>
<td>O</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabies</td>
<td>Im</td>
<td>X</td>
<td>(X)</td>
<td></td>
</tr>
<tr>
<td>Rotavirus</td>
<td>O</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smallpox</td>
<td>Sc</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tick-borne encephalitis</td>
<td>Im</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Varicella-Zoster</td>
<td>Im</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yellow fever</td>
<td>Sc</td>
<td>X</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Im – Intramuscular, Sc – subcutaneous, O – oral

Appendix 1 shows the virus vaccines currently on the market. The table details the name of the virus, route of administration and the vaccine type. Details of commercially available vaccines used in the UK can be found in the British National Formulary (www.bnf.org, September 2009)