INTERACTIONS OF HIV-1 WITH ANTIGEN PRESENTING CELLS

Timothy John Hewson BSc (Hons) GIBiol

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University of Edinburgh

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ABSTRACT

Human immunodeficiency virus (HIV) infects about 40 million people worldwide. HIV is the causative agent of acquired immunodeficiency syndrome (AIDS). AIDS is characterised by a progressive decline in protective immunity that leads to opportunistic infection and eventually death. Although HIV-1 causes a decline in $CD4^+$ T-cell number and this undoubtedly contributes to the general immune deficit of AIDS, $CD4^+$ T-cell loss does not completely explain the pathogenesis of AIDS. Death and anergy of uninfected T-cells is observed in AIDS, as are deficits in innate and specific immunity. Antigen presenting cells (APCs, including macrophages and dendritic cells, DCs, which are infectable by M-tropic strains of HIV-1 via the CCR-5 chemokine receptor) play a key role in orchestrating innate and adaptive immune responses and controlling T-cell activities including activation, anergy, deletion, tolerisation and memory by the provision of appropriate signals. APC dysregulation results in deficits of innate and adaptive immune responses. It is known that HIV-1 can cause APC dysregulation; this thesis examines some mechanisms by which this might occur.

The HIV-1 envelope glycoprotein gp120 mediates HIV-1 infection by binding to target cells via CD4 and CCR-5 and is focussed on throughout this work. Because gp120 is found on the surface of HIV-1 and dissolved in the serum of HIV-1 infected patients, it has the ability to disrupt the function of both infected and uninfected APCs.

Data in this thesis demonstrate that gp120 causes a decline of cell-surface CD4 from human macrophages *in vitro*. A mechanism for this loss is proposed based on observations that it is significantly more substantial when CCR-5-binding gp120, derived from M-tropic HIV-1 is used as opposed to CXCR-4-binding gp120. CD4 loss is absent from macrophages that fail to express surface CCR-5 due to homozygosity for the naturally occurring *ccr5Δ32* mutation. It appears that CD4 loss by this novel CCR-5-dependent mechanism requires cross-linking of CCR-5, CD4 and gp120 at the cell surface leading to receptor-mediated endocytosis of this protein complex. Confocal microscopy was used to visualise these endocytosed proteins inside macrophages and RT-PCR was used to investigate transcriptional regulation of CD4 and CCR-5 recovery. Endocytosis of the protein complex may change antigen presentation efficiencies. Possible implications for protective- and auto-immunity are discussed.

This thesis also presents evidence that pre-treatment with gp120 leads to reduction in an APC's ability to stimulate antigen-specific proliferation of a T-cell line. Because this effect is not dependent on the tropism of the HIV-1 strain from which the gp120 is derived, an alternative mechanism to CD4-loss was sought. The hypothesis that APC dysfunction is due to HIV-1 subversion of physiological mechanisms involving prostaglandin and the Notch signalling pathway, leading to inappropriate tolerance induction, was examined. Treatment of macrophages and DCs with gp120 caused the transcriptional up-regulation of genes involved in the Notch pathway including Notch ligands, the presence of which on an APC has previously been shown to abrogate T-cell activation by the induction of an anergic phenotype.

Preventing HIV-1 infection of APCs and the subsequent dysregulation of immune responses is a therapeutic goal. Branched, synthetic peptides based on discontinuous epitopes of gp120 and previously demonstrated to disrupt binding to CD4 and CCR-5 are shown to protect macrophages from infection with M-tropic $HIV\text{-}1_{BAL}$. Possible refinements to peptide structure and their utility as anti-HIV-1 therapeutics or vaccines are discussed.

DECLARATION

I hereby declare that the work presented in this thesis is my own, except where stated in the text. The work has not been submitted in any previous application for a degree.

Timothy John Hewson

We have our philosophical persons, to make modern and familiar, things supernatural and causeless.

All's Well That Ends Well, II, iii

With love to Jill, Rob and my parents

CONTENTS

ABREVIATIONS

Abbreviation	Full Form
19HO-PGE	19-hydroxy-PGE
3.7	gp120 based peptide (see chapter 6)
[^{3}H]dThd	tritiated deoxythymidine triphosphate
A	adenine (base) / (deoxy)adenosine (nucleoside) / (deoxy)adenylate (nucleotide), Ampere
Ab	antibody
AC1.1	a Der P II specific T-cell line
ADE	antibody dependent enhancement (of infection)
AICD	activation-induced cell death
AIDS	acquired immune deficiency syndrome
allele	allelomorph(ic form of a polymorphic gene)
AP-1	(transcription) activator protein 1
APC	antigen presenting cell
ARC	AIDS-related complex
ARV	AIDS-related retrovirus (now HIV-1)
AS	anti-sense (PCR primer)
AZT	3'-azido-3'-deoxythymidine
b	base
BALB/C	inbred mouse strain
Bcl	B-cell CLL / lymphoma (oncogene)
BMP	bone morphogenetic protein
bp	base pair
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin (fraction V)
C	cytosine (base) / (deoxy)cytidine (nucleoside) / (deoxy)cytidylate (nucleotide), complement component (C1 to C9), constant (domain of protein), carboxyl (terminal of peptide)

CAMP	cyclic adenosine monophosphate
CBF-1	C-promoter binding factor 1 / core binding factor 1
CCR	CC (α) chemokine receptor
CD	cluster of differentiation (HLDA designation)
CDC	(USA) Center for Disease Control and Prevention
cDNA	complimentary DNA
CDR	complimentarity determining region
CFA	complete Freund's adjuvant
Ci	Curie
CKR	chemokine receptor
CMV	cytomegalovirus
Con A	concanavalin A
COX	cyclooxygenase
CPM	counts per minute
CSF	colony-stimulating factor
CTL	cytotoxic T lymphocyte / killer T-cell
CTLA-4	cytotoxic T lymphocyte antigen (CD 152)
CXCR	CXC (β) chemokine receptor
D	Dalton
DC	dendritic ceil
DC-SIGN	DC-specific, ICAM-3 grabbing non-integrin
Dde	4,4-dimethyl-2,6-dioxocyclohex-1-ylidine
Der p 1	*Dermatophagoides pteronyssinus* (house dust mite) allergen 1
DMSO	dimethyl sulphoxide
DNA	deoxyribo(se) nucleic acid
dNTP	deoxynucleoside triphosphate
DSL	Delta / Serrate / Lag-2 (motif)
dT	deoxythymidine
EAE	experimental autoimmune / allergic encephalitis (animal model of MS)
EBNA	Epstein-Barr (virus) nuclear antigen

EBV	Epstein-Barr virus
EDTA	ethylene di-amino tetra-acetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immuno-sorbant assay
env	HIV envelope gene
ER	endoplasmic reticulum
EU	European Union, exposed-uninfected
F(ab')	antigen-binding fragment of Ig
FAM	carboxyfluorescein-N-hydroxysuccinimide
FasL	Fas ligand
Fc	crystalline fragment of Ig
FcR	Fc receptor
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FMDV	foot and mouth disease virus
F-moc	9-fluorenylmethoxycarbonyl
F.S.	forward light scatter
ft	foot
g	gram
g	acceleration due to gravity (~10 m s^{-2})
G	guanine (base) / (deoxy)guanosine (nucleoside) / (deoxy)guanylate (nucleotide), gap phase of cell cycle (e.g., G_0, G_2), GTP binding (e.g., G-protein)
gag	HIV group-specific antigen gene
GAP-DH	glyceraldehyde 3-phosphate dehydrogenase
GC-1 / -2	gp120 based peptide (originally made by Garry Cotton)
G-CSF	granulocyte CSF
GLP	good laboratory practice
GM-CSF	granulocyte-monocyte/macrophage CSF
gp	glycoprotein
Grb	growth factor receptor-bound protein
Gy	Gray

H-9	a T-cell line
HA	(influenza) haemagglutinin
HA1.7	an influenza haemagglutinin specific T-cell line
HAART	highly active anti-retrovirus therapy
HeLa	a cervical carcinoma cell line
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HES	hairy and enhancer of split
HI-NABHS	heat inactivated normal blood-group AB human serum
HIV-1 / -2	human immunodeficiency virus -type 1 / -type 2
HLA	human leukocyte antigen
HLDA 1-7	human leukocyte differentiation antigen (workshops 1-7)
hr	hour
HTLV-III	human T-cell leukaemia virus type III (now HIV-1)
HWE	Hardy-Weinberg equilibrium
IBD	inflammatory bowel disease
ICAM	intercellular adhesion molecule
IFN	interferon
IgG, IgM etc	immunoglobulin class G, M etc
IH-1 / -2	gp120 based peptide (originally made by Ian Heslop)
IL	interleukin
IMDM	Iscove's modified Dulbecco's medium
IRF	IFN regulatory factor
ISRE	IFN-stimulated response element
JNK	c-Jun N-terminal kinase
l	litre
L	ligand
LAK	lymphokine-activated killer (cell)
LAV	lymphadenopathy-associated virus (now HIV-1)
LC	Langerhans cell
LNC	lymph node cell
LNR	Lin-12 / Notch repeat (motif)
LPS	lipopolysaccharide

LTR	long terminal repeat
m	meter
M	molar
mAb	monoclonal antibody
MAP	mitogen activated protein
MBP	mannose binding protein, myelin basic protein
M-CSF	monocyte/macrophage CSF
MHC	major histocompatibility complex
min	minute
MIP	macrophage inflammatory protein (chemokine)
MM6	mono/mac 6 (cell line)
MnIX	mean fluorescence intensity of X
MOI	multiplicity of infection
mol.	mole
Mona	monocytic adaptor
MRC	(UK) Medical Research Council
mRNA	messenger RNA
MS	multiple sclerosis
N	nucleotide / nucleoside / base (e.g. in dNTP), amino terminal (of peptide)
NCR	Notch cytokine response (protein region)
N.D.	not determined
nef	HIV early regulatory gene
NFAT	nuclear factor of activated T-cells
NF-κB	nuclear (transcription) factor κB
NIBSC	(UK) National Institute for Biological Standards and Controls
NK	natural killer (cell)
NOD	non-obese diabetic
NSI	non-SI
OVA	(hen egg) ovalbumin
P	probability value
PBMC	peripheral blood mononuclear cell

PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin, Perkin Elmer (trademark)
PEST	proline, glutamic acid, serine, threonine (protein motif rich in these amino acids)
PG	prostaglandin
PGE	prostaglandin E
pGEM	Promega molecular weight marker
PGL	persistent generalised lymphadenopathy
pH	$-\log_{10}[H^+]$
PI	propidium iodide
pol	HIV polymerase / integrase / endonuclease gene
r	ribosomal
R	receptor, rectus (clockwise)
R5-tropic	CCR5-tropic
RANTES	regulated upon activation, normal T-cell expressed and secreted
RBPJκ	recombination recognition sequence binding protein at the Jκ site (synonym of CBF-1)
rev	HIV early regulatory gene
RNA	ribo(se)nucleic acid
ROX	carboxyxrhodamine-N-hydroxysuccinimide
R-PE	R enantiomer of PE
rpm	revolutions per minute
RPMI 1640	Roswell Park Memorial Institute (culture medium number) 1640
rRNA	ribosomal RNA
RT	reverse transcript / transcription / transcriptase, room temperature, respiratory tract
RT-PCR	reverse transcript directed PCR
s	second
S	Svedberg (sedimentation coefficient), sense (PCR primer)

SDF	stromal-derived factor
SHIV	SIV / HIV-1 artificially produced chimeric virus
SI	syncytium inducing (virus strain)
SIV	simian immunodeficiency virus
SNBTS	Scottish National Blood Transfusion Service
SPE	seminal plasma extract
S.S.	side (90°) light scatter
STAT	signal transducer and activator of transcription
T	thymine (base) / deoxythymidine (nucleoside) / deoxythymidylate (nucleotide)
TAMRA	carboxytertramethylrhodamine-N-hydroxysuccinimide
TAN-1	truncated allele of *notch-1* (oncogene)
TAP	transporter associated with antigen processing
Taq	*Thermus aquaticus* DNA polymerase
tat	*trans*-acting transcriptional activator
TBE	tris / boric acid / EDTA buffer
Tc	T cytotoxic (killer cell)
$TCID_{50}$	50% tissue culture infectious dose
TCLA	T-cell line adapted (HIV strains)
TCR	T-cell receptor
TGF	transforming growth factor
Th	T helper (cell)
T_m	melting (denaturing) temperature of nucleic acid
TM	TaqMan® (real time PCR probe)
TNF	tumour necrosis factor
Tr	T regulatory (cell)
tris	tris (hydroxymethyl) aminomethane
TRITC	tetramethyl rhodamine isothiocyanate
Ts	T suppressor (cell)
U	uracil (base) / uridine (nucleoside) / uridylate (nucleotide), (WHO validated international activity) unit
UNAIDS	United Nations / WHO AIDS programme

UNG	Uracil N-glycosylase
UV	ultra violet (radiation)
V	Volts, variable (domain of protein)
vif	viral infectivity factor
vpr	viral protein R
vpu	viral protein U
VSV	vesicular stomatitis virus
v/v	volume per unit volume
WHO	World Health Organization
wt	wildtype
w/v	weight (mass) per unit volume
X4-tropic	CXCR4-tropic

AMINO ACID ABREVIATIONS

Single letter abbreviation	**Three letter abbreviation**	**Full name**
A	Ala	alanine
C	Cys	cysteine
D	Asp	aspartic acid
E	Glu	glutamic acid
F	Phe	phenylalanine
G	Gly	glycine
H	His	histidine
I	Ile	isoleucine
K	Lys	lysine
L	Leu	leucine
M	Met	methionine
N	Asn	asparagine
P	Pro	proline
Q	Gln	glutamine
R	Arg	arginine

S	Ser	serine
T	Thr	threonine
V	Val	valine
W	Trp	tryptophan
X		any amino acid
Y	Tyr	tyrosine

GENES OF THE NOTCH PATHWAY

Many of the genes involved in Notch signalling (chapter 5) were independently identified in different species. This means that a large number of names, and acronyms have been used to describe each gene and its protein product. The list below gives the principle genes discussed in this thesis, synonyms and names of orthologues that will be encountered in the literature. Vertebrate names have mainly been used except were this would result in the use of unwieldy and rarely used acronyms. Note that the situation, especially in mammals, is further complicated by homologues which may or may not be functionally equivalent (e.g., notch-1, -2, -3 etc). The convention of giving gene names in italics and corresponding protein names in upright characters with uppercase initial letter has been followed.

CBF-1

CSL proteins (collectively for CBF-1, Su(H) and LAG-1)

KBF-2 (mammal)

Lag-1 (*Caenorhabditis elegans*)

RBP-Jκ (mammal)

suppressor of hairless / Su(H) (*Drosophila melanogaster*)

delta

DeltaD (Zebra fish)

dll / delta-like (mouse)

lag-2 (*C. elegans*)

X-delta (*Xenopus laevis*)

deltex

- *FXI-TI* (mammalian)
- *dx*

Grb-2

- *Ash*
- *Sem-5* (*C. elegans*)

HES

- *enhancer of split / E(spl) (D. melanogaster)*
- *Hairy (HES-1)*
- *HRY (*human *HES-1)*

notch.

- *abruptex* (*D. melanogaster* gain of function allele)
- *glp-1* (*C. elegans*)
- *int-3* (mouse *notch-4*, mammary tumour oncogene)
- *lin-12* (*C. elegans*)
- *motch* (mouse *notch-1*)
- *split* (*D. melanogaster* allele)
- *TAN-1* (truncated allele of human *notch-1*, leukaemia oncogene)
- *xotch* (*X. laevis*)

serrate

- *jagged*

Ankyrin repeat (protein motif)

- Ankyrin-related repeats
- cdc10 repeats
- SW16 repeats

CHAPTER 1

INTRODUCTION

Human immunodeficiency viruses

AIDS

> *"Everybody knows that pestilences have a way of recurring in the world; yet somehow we find it hard to believe in ones that crash down on our heads from a blue sky"*
>
> Albert Camus, The Plague, 1948 (Camus, 1989)

In 1981 a new disease originally dubbed GRID (gay-related immunodeficiency disease) was reported amongst gay men in San Francisco (Anon., 1981). The name was changed to AIDS (acquired immune deficiency syndrome) in August 1982 in recognition that it is not just a disease of gay men (Harris *et al.*, 1983). AIDS is a disease caused and characterised by the breakdown of the host immune system leading to the appearance of opportunistic infections and malignancies normally kept at bay by a healthy immune system (see Castro *et al.*, 1993, and table 1.1 for a list of AIDS defining conditions).

Remarkably quickly after the discovery of AIDS, the causative retrovirus agent, now called type 1 human immunodeficiency virus (HIV-1), was isolated from the lymph node of a patient (BarréSinoussi *et al.*, 1983). A second related retrovirus, HIV-2 was isolated a few years later from patients in West Africa with AIDS-like symptoms (Clavel *et al.*, 1986; Blanc, 1986; Clavel *et al.*, 1987). HIV-2 is more closely related than HIV-1 to subsequently identified simian immunodeficiency viruses (SIV, Kanki *et al.*, 1986; Franchini and Bosch, 1989). Since its discovery HIV-2 has remained mainly localised to West Africa where it causes a less severe disease than HIV-1. The majority of HIV infections world-wide are by HIV-1 and this virus is the main focus of HIV research.

HIV-1 transmission

HIV-1 is transmitted by exchange of bodily fluids (VanDerGraaf and Diepersloot, 1986; Tomaso *et al.*, 1995). This may be by homosexual (Weller *et al.*, 1987) or heterosexual activity (Vilmer *et al.*, 1984; Clotet *et al.*, 1986; Alcami and Koszinowski, 2000), from mother to foetus during pregnancy or birth (Vilmer *et al.*, 1984; Coulaud *et al.*, 1986), by the use of non-sterile medical equipment especially syringes in medical procedures or intravenous drug abuse (Desjarlais *et al.*, 1988) or by engraftment of infected tissue (Gluckman *et al.*, 1985), blood or blood products (Melief and Goudsmit, 1986; Alter, 1987). Although HIV-1 has been detected in urine and saliva and can be transmitted by oral-genital contact (Kaplan *et al.*, 1985; Scully and Porter, 2000), there have been no substantiated reports of transmission due to normal social contact (Operskalski and Mosley, 1986; Philipson and Lorincz, 1986; McDonald and Rogers, 1986; Friedland *et al.*, 1986; Friedland *et al.*, 1986), nor have biting insects been implicated in transmission. The mode of transmission may involve the transfer of free virions or HIV-1 infected cells (Fan and Peden, 1992; Zhu *et al.*, 1995; Zacharopoulos *et al.*, 1997).

Progression to AIDS

Initial (acute) infection with HIV-1 results in clinical symptoms within one to three weeks in at least half of those newly infected. These symptoms are similar to influenza infection or mononucleosis, along with a non-pruritic macular erythematous rash (Fox *et al.*, 1987). Shortly after acute infection, most patients undergo seroconversion. This is followed by a period of clinical latency, which may last from three to more than 15 years (Buchbinder *et al.*, 1994), before AIDS develops and the patient eventually dies of multiple infections and/or malignancies (Castro *et al.*, 1993). Progression to AIDS is accompanied by loss of $CD4^+$ T-lymphocytes with symptoms being noted at blood levels less than 500-cells/μl. AIDS related complex (ARC) is a term used to indicate the presence of some immune system abnormality and low-grade clinical disease (such as malaise) in HIV positive patients who have yet to develop to 'full-blown AIDS' as indicated by the presence

of a defined set of infections or cancers (Castro *et al.*, 1993) See table 1.1 for the CDC clinical classification of HIV-1 disease.

Although the vast majority of those who are infected with HIV-1 will develop AIDS there is mounting evidence that some people are able to live with the virus for extended periods of time without developing clinical disease. Such individuals are termed 'long term non-progressors' or 'long term survivors', although only time will tell if this group will also succumb to disease (Buchbinder *et al.*, 1994). Factors which affect the rate of progression to AIDS (for review see Levy, 1994), include age (most HIV-1 infected infants progress relatively slowly, Blanche, 1996; Blanche *et al.*, 1997; Blanche, 1996), general health (the presence of other infections may speed progression to AIDS, Wahl and Orenstein, 1997) and lifestyle (tobacco smoking, Twigg *et al.*, 1994, alcohol, Bagasra *et al.*, 1993a, and drug use, Bagasra and Pomerantz, 1993b, may all speed progression). Differences in the infecting HIV-1 strain and the host immune response are probably also important in disease progression rates.

	Clinical category		
$CD4^+$ T-cell category	(A) asymptomatic, acute (primary) HIV infection or PGL	(B) Symptomatic, not (A) or (C) conditions	(C) AIDS indicator conditions
(1) ≥500/μl (2) 200 499/μl (3) <200/μl – AIDS	Asymptomatic HIV infection; persistent generalised lymphadenopathy (PGL); acute (primary) HIV infection with accompanying illness or history of acute HIV infection	Bacillary angiomatosis; candidiasis, oropharyngeal (thrush); candidiasis, vulvovaginal; persistent, frequent, or poorly responsive to therapy; cervical dysplasia (moderate to severe) / cervical carcinoma *in situ*; constitutional symptoms, such as fever (28.5°C) or diarrhoea lasting > 1 month; hairy leukoplakia, oral herpes zoster (shingles), involving at least two distinct episodes or more than one dermatome; idiopathic thrombocytopenic purpura; listeriosis; pelvic inflammatory disease, particularly if complicated by tubo-ovarian abscess; peripheral neuropathy	Candidiasis of bronchi, trachea or lungs; candidiasis, oesophageal; cervical cancer, invasive; coccidoidomycosis, disseminated or extrapulmonary; crytoccosis, extrapulmonary; cryptosporidiosis, chronic intestinal (> 1 month's duration); cytomegalovirus disease (other than liver, spleen or nodes); cytomegalovirus retinitis (with loss of vision); encephalopathy, HIV-related; herpes simplex; chronic ulcer(s) (>1 month's duration); or bronchitis, pneumonitis, or oesophagitis; histoplasmosis, disseminated or extrapulmonary; isosporiasis, chronic intestinal (> 1 month's duration); Kaposi's sarcoma; lymphoma, Burkitt's (or equivalent term); lymphoma, immunoblastic (or equivalent term); lymphoma, primary, or brain; *Mycobacterium avium* complex or *M. kansasii*, disseminated or extrapulmonary; *Mycobacterium*, other species or unidentified species, disseminated or extrapulmonary; *Pneumocystis carinii* pneumonia; pneumonia, persistent; progressive multifocal leukoencephalopathy; salmonella septicaemia, recurrent; toxoplasmosis of brain; wasting syndrome due to HIV

(caption over)

Table 1.1 (previous page). CDC revised classification system for HIV infection. Persons with category C conditions or category 3 $CD4^+$ T-cell counts are reported as AIDS cases to the surveillance authorities in most countries.

Table taken from Castro *et al.*, 1993

Type 1 human immunodeficiency virus (HIV-1)

HIV-1 is a lentivirus of the family *Retroviridae*. Lentiviruses have a long incubation period and several are associated with the haematopoeitic and immune systems (Levy, 1994). Related mammalian lentiviruses include feline immunodeficiency virus (FIV, Yamamoto *et al.*, 1987), and simian immunodeficiency virus (SIV, Kanki *et al.*, 1986; Guo *et al.*, 1987; Fukasawa *et al.*, 1988). Both FIV and SIV have been used as models for HIV-1 infection with some success, although infection of animals with these viruses, or the infection of monkeys with HIV-1 or HIV-2 does not resemble human infection with HIV-1 in all aspects. Small animals are not infectable by HIV-1 or HIV-2 (Morrow *et al.*, 1987). HIV-1 has the structure characteristic of a lentivirus (figure 1.1); this consists of a truncated cone shaped core, which contains two copies of a single-stranded RNA genome (figure 1.2) and the enzyme reverse transcriptase. A protein matrix enclosed in a lipid envelope, which bears 72 knobs of the envelope glycoprotein gp160 surrounds the core. Gp160 consists of an external gp120 peptide and a gp41 transmembrane component.

Because HIV-1 utilises a reverse transcriptase enzyme with a low fidelity (Preston *et al.*, 1988) it has a very high mutation rate and consequently much genetic diversity. Viral diversity exists on several levels but is particularly important in the major immunogenic protein gp120, which has several hypervariable (V) regions.

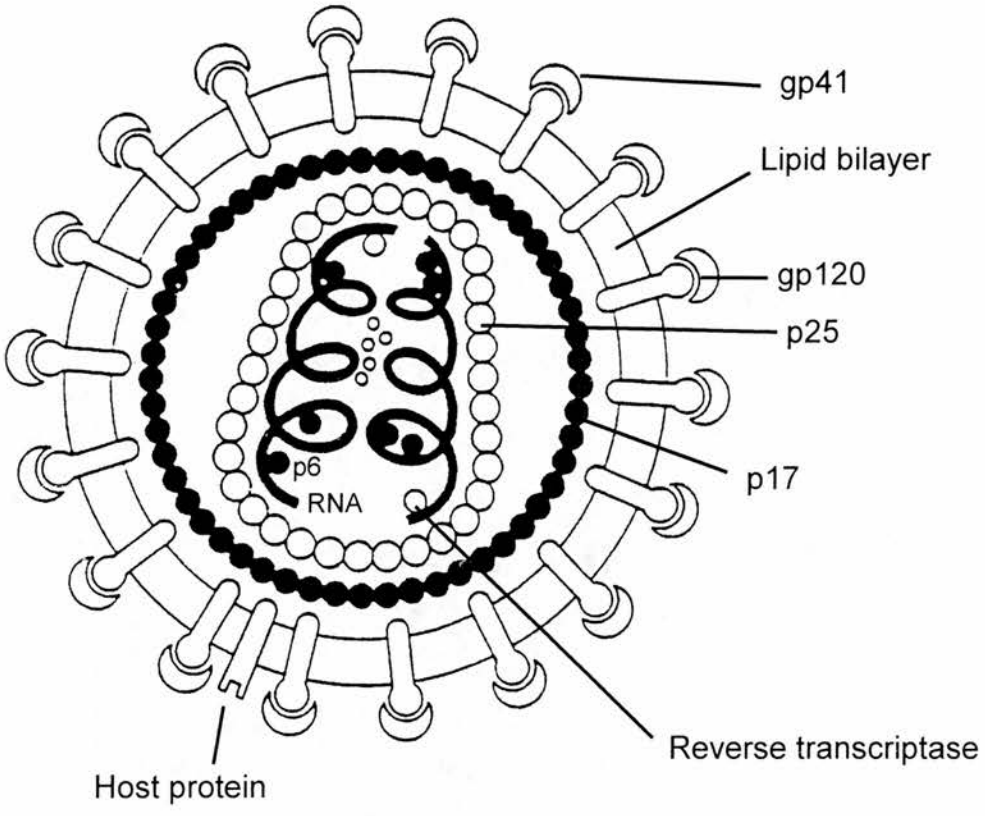


Figure 1.1. Diagram of HIV-1 structure. HIV-1's genomic RNA and reverse transcriptase (encoded by *pol*) is packaged inside a truncated-cone shaped nucleocapsid made of p25 (also known as p24) protein subunits encoded by the *gag* gene. HIV-1 is enveloped in a host cell derived lipid bilayer, which is supported by a matrix of p17 subunits which are also *gag* encoded. Exposed on the surface of the virion is gp41 and gp120, which are non-covalently associated cleavage products of the gp160 protein encoded by the *env* gene. Host membrane proteins such as MHC molecules and CD4 may also be found on the virion envelope.

Figure adapted from Heslop, 1997. © I Heslop, 1997.

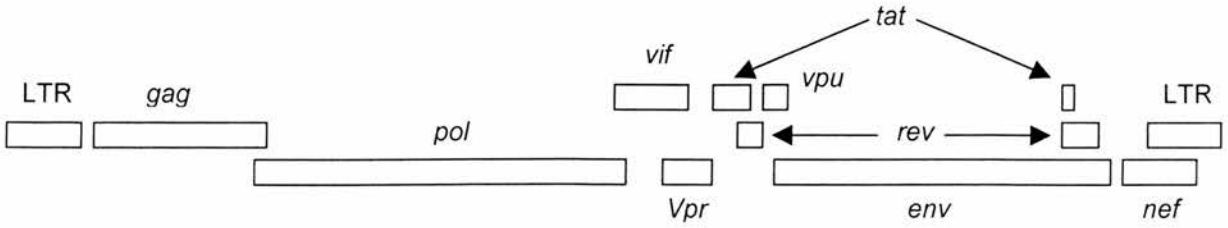


Figure 1.2. The genomic organisation of HIV-1. The approximately 10kb genome is flanked 3' and 5' by LTRs (long terminal repeats) which act as HIV-1's promoter. Several of the genes are overlapping and *tat* and *rev* are discontinuous. Full length, sub-genomic and spliced mRNA are therefore required for complete translation of the viral genome. *Gag* encodes p25, p17, p9 and p6 (figure 1.1). *Pol* codes for reverse transcriptase and RNase H. *Env* encodes gp160. Other genes are involved in various stages of the HIV-1 replication cycle.

Figure redrawn from Levy, 1994. © American Society for Microbiology, 1994.

HIV-1 clades

The comparison of genomic sequences encoding the V3 region of gp120 has allowed twelve HIV-1 subtypes (clades) to be identified (A-J, plus the O grouping for outliers and the N (New) grouping from Cameroon, Simon *et al.*, 1998). The clades show broad but distinct geographic ranges. Clade A is prominent in central Africa, clade B in north America and Europe, clade C in South Africa and India, clade D in central Africa, clade E in Thailand and clade F in south America. Such a distribution pattern could be due to patterns of spread, to differences in host immunology brought about by the geographic distribution of selective pressure from other pathogens, and/or to differences in the predominant transmission mode. The HIV-1 epidemic in Thailand is largely due to heterosexual transmission. There is some suggestion (SotoRamirez *et al.*, 1996; Pope *et al.*, 1997) that the clade E virus, common in Thailand, is especially well suited to this transmission route as it is able to replicate better than clade B viruses in Langerhans cells, which have been implicated in transmission across the vaginal mucosa. Clade B viruses may be better adapted to the homosexual and intravenous transmission routes most common in North America and Europe.

CD4 and HIV-1

The main cell surface receptor for HIV-1 is the CD4 molecule (Dalgleish *et al.*, 1984); and $CD4^+$ T-cells are a major cellular target for infection. The gp120 envelope glycoprotein on the virion surface binds to CD4 and then to a chemokine receptor (usually CXCR-4 or CCR-5, D'Souza and Harden, 1996) on the target cell. After CD4 and CCR-5 binding, a conformational change in gp120 allows a fusogenic region of gp41 to become exposed and mediate the fusion of the viral envelope with the target cell membrane (Demaria and Bushkin, 1996; Pereira *et al.*, 1997; Jones *et al.*, 1998; Ji *et al.*, 1999; Mashikian *et al.*, 1999). CD4 is an important molecule in the immune system. In addition to acting as an MHC class II coreceptor on T-cells (Janeway and Travers, 1996), it is also a receptor for interleukin-16 (IL-16, originally called lymphocyte chemoattractant factor) on several immune cell types (Center *et al.*, 1995). CD4 is also found on activated cells of the monocyte, macrophage and dendritic lineages; these cells have a role as antigen presenting cells (APCs) and are infected by HIV-1 both *in vitro* and *in vivo* (BarréSinoussi, 1988; Spira *et al.*, 1996).

Cellular tropism of HIV-1

The crystal structure of the CD4 binding site has recently been published (Kwong *et al.*, 1998) and involves several conserved residues in the gp120 molecule. However, it had long been suspected that HIV-1 required an additional, secondary, receptor in order to infect cells. Research since 1995 (for reviews see D'Souza and Harden, 1996, and Berger, 1997) has identified the secondary receptor as a member of the chemokine receptor family (see figure 1.4). At least 10 different chemokine receptors have been identified. HIV-1 can utilise several of these molecules as a secondary receptor but most commonly uses CCR-3, CCR-5 and/or CXCR-4 (Zhang and Moore, 1999; for a review of chemokine and chemokine receptor function and nomenclature see Baggiolini *et al.*, 1997). The type of chemokine receptor which the virion is able to use depends on the sequence of its gp120. The V3 loop of gp120 is particularly important in determining coreceptor usage (Fouchier *et al.*, 1992). The viral DNA encoding this region is highly variable between viral strains and also mutates during the course of infection allowing the virus to change its coreceptor

usage, and therefore phenotype, with time (Schuitemaker *et al.*, 1992). However, it has recently been shown that conserved regions of gp120 are also important in binding to chemokine receptors (Verrier *et al.*, 1997). The conformation of the V3 loop changes on CD4 binding (Demaria and Bushkin, 1996); this may allow previously hidden conserved residues access to chemokine receptors (see figure 1.3 and Jones *et al.*, 1998).

Because of a differential distribution of chemokine receptors between cell types and a poorly understood apparent differential availability of these receptors for gp120 binding (Yi *et al.*, 1999), the type of receptor that a particular gp120 is able to bind to influences the cellular tropism of the virion (Moore *et al.*, 1997; Tscherning *et al.*, 1998). Most primary isolates of virus from patients, at least during the early stages of infection, are macrophage- (M-) tropic, can infect both macrophages (including alveolar macrophages, Park *et al.*, 1999) and T-cells, and use the CCR-5 coreceptor, which is expressed on both cell types. Lab adapted strains grown for many passages on T cell lines use CXCR-4 as a coreceptor. Although CXCR-4 is present on both macrophages and T-cells, most T-cell line adapted viruses can only use the CXCR-4 on T-cells or T-cell lines, the CXCR-4 coreceptor on macrophages appears to be unavailable for binding in this case (Yi *et al.*, 1999). Dual-tropic viral strains (Doranz *et al.*, 1996) which can use both CCR-5 and CXCR-4 are probably more common in patients than solely CXCR-4 utilising T-tropic strains. Dual tropic strains are able to infect both macrophages and T-cells; interestingly, macrophage CXCR-4 appears to be available as an entry coreceptor for some dual-tropic HIV-1 strains (Yi *et al.*, 1999). Dendritic cells (DCs) behave similarly to macrophages in respect to HIV-1 tropism, presumably because of their similar chemokine receptor expression. An alternative and complementary classification of HIV-1 tropism refers to the chemokine receptor used as a coreceptor rather than the cellular range of infectable targets. Under this scheme HIV-1 may be classified as R5 (CCR-5), X4 (CXCR-4) or R5X4 (CCR-5 and CXCR-4) tropic. It is important to realise that the designation of viral strains to particular tropisms is only an approximation of reality (Stent *et al.*, 1997b) and that the two classification schemes outlined above do not always equate with each other (Yi *et al.*, 1999). Tropisms overlap and the infectability of a cell depends on its activation state as well as its phenotype. Almost

all HIV-1 strains enter and replicate in activated T-cells if added to cultures at sufficiently high concentrations (Moore *et al.*, 1997), and there are reports of certain TCLA SI HIV-1 strains using CXCR-4 to infect macrophages, including CCR-5 deficient macrophages (Simmons *et al.*, 1998; Verani *et al.*, 1998). HIV-1 is able to target a fairly wide range of cell types for infection (see Levy, 1994, for a comprehensive list) and can use a variety of coreceptors to do this. Neuronal and microglial HIV-1 infections are of importance in the aetiology of AIDS related dementia; CCR-5 is the main coreceptor used to infect these cell types (Donaldson *et al.*, 1994; He *et al.*, 1997; Albright *et al.*, 1999). HIV-1 may also disrupt the function of cells without infecting them or even binding to them.

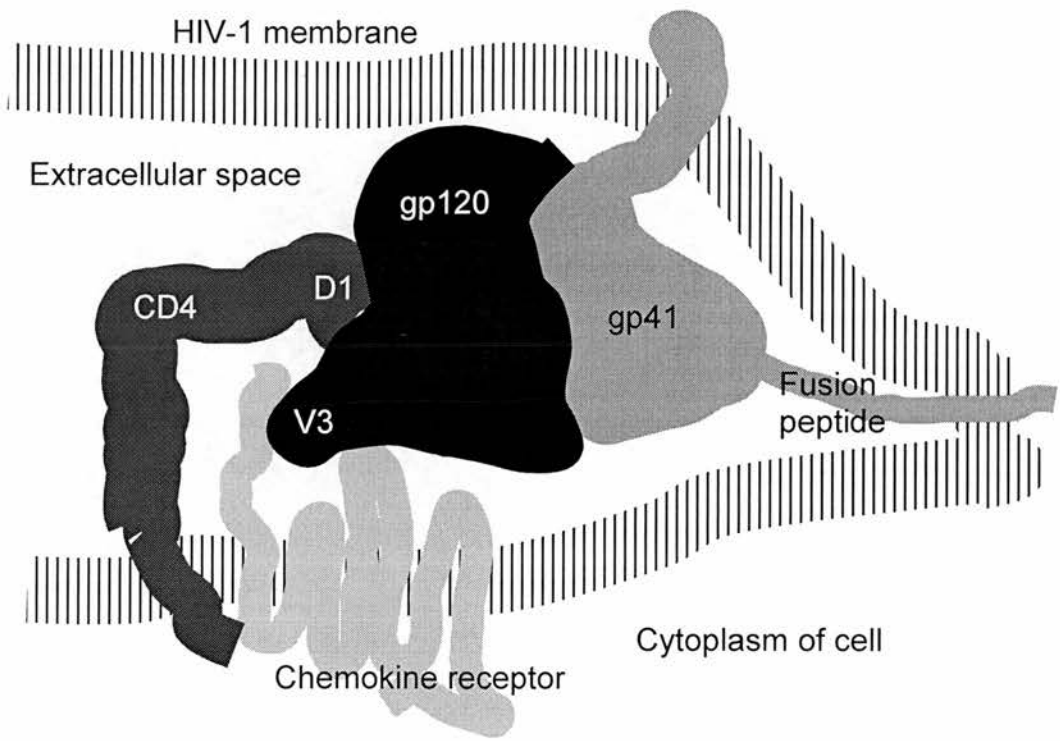


Figure 1.3. Interactions leading to HIV-1 fusion with target cell. CD4 binds to gp120 and causes exposure of the V3 loop, allowing a chemokine receptor to interact with the gp120 / gp41 / CD4 complex leading to the exposure of a previously hidden gp41 fusion peptide. The hydrophobic fusion peptide induces membrane attraction and destabilisation, resulting in the viral envelope and plasmalemma fusing.

Figure redrawn from Dimitrov, 1996. © Nature America 1996.

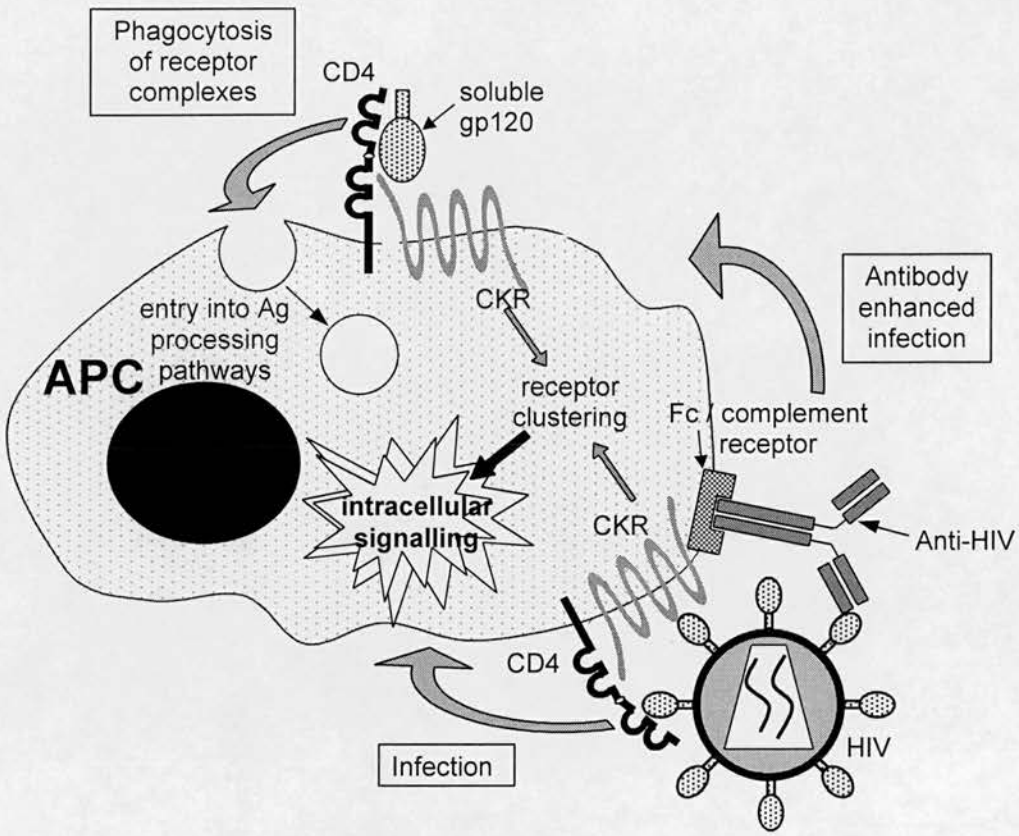


Figure 1.4, HIV-1 interactions with APC cell surface receptors. HIV-1 and its shed surface protein gp120 are able to interact with a number of receptor molecules on APCs. Gp120 has binding sites for CD4 and chemokine receptors (CKRs) such as CCR-5. It can also interact with Fc or complement receptors via anti-HIV-1 antibodies. Outcomes of HIV-1 proteins binding to APC receptors include infection of the cell by HIV-1 (a process which can be enhanced by antibodies to HIV-1, antibody enhanced infection, Toth *et al.*, 1994), and phagocytosis of receptor complexes bound to soluble gp120, leading to the loss of receptors such as CD4 and possible entry of the receptor / gp120 complex into antigen processing pathways. It should be noted that all of the HIV-1 receptors are linked to intra-cellular signalling pathways, which may be activated on binding HIV-1 or gp120, and that this could lead to disruption of the cell's function.

Figure adapted from Hewson *et al.*, 1999. Artwork by Nazir Lone.

Antigen presenting cells (APCs)

Introduction

Recognition of antigen by T-cells is a vital event in the immune system. It is involved in the initiation of protective humoral and cell-mediated immunity and, via antigen specific induction of anergy (Frauwirth *et al.*, 2000; Oda *et al.*, 2000; Bouhdoud *et al.*, 2000) and regulatory cells (Chen *et al.*, 1994; Sakaguchi *et al.*, 1996; Kumar and Sercarz, 1998), the induction of immune tolerance. Unlike B-cells, T-cells are unable to respond to native antigen. T-cells require that antigen is first processed into peptides of suitable length and presented to the T-cell receptor whilst bound to an MHC molecule (Shimojo *et al.*, 1990; Nygard *et al.*, 1992; Marshall *et al.*, 1994). All nucleated cells have the ability to present peptides in MHC class I molecules to $CD8^+$ T-cells. However, $CD4^+$ T-cells need peptide presented by MHC class II, which is only expressed on a limited range of cell types. In order to be activated, naive T-cells must receive additional, co-stimulatory signals, as well as properly presented peptide (see below for details). Professional APCs have a vital role in the immune system. As well as acting as effectors of the innate (phagocytic) immune system (Janeway and Travers, 1996), APCs link the innate and adaptive immune systems by efficiently presenting T-cells with processed antigen peptides bound to MHC class I and II, and by giving T-cells additional, non-antigen-specific, signals (co-stimulation, cytokines and others) to direct the T-cell in mounting an appropriate response (Kuribayashi *et al.*, 1997). Any disease that disrupts APCs therefore has the potential to disrupt both innate and acquired immune responses.

Antigen presenting cell types

Although B-cells are able to act in many ways like professional antigen presenting cells, the term APC is often used to mean macrophages and DCs. Haematopoiesis begins with pluripotent stem cells (Till and McCulloch, 1961) in the bone marrow of adults or in the foetal liver, which give rise to all blood cell types including monocytes which circulate in the blood. Upon entering tissue, monocytes differentiate into macrophages or DCs. Both these cell types are highly heterogeneous in terms of their MHC and co-stimulatory molecule expression,

cytokine profiles, phagocytic and migratory abilities. The local tissue microenvironment is able to maintain specialised sub-types of macrophages and DCs such as microglia in the brain (monocyte / macrophage-like) and Langerhans cells (LCs) in the mucosa and skin (DC like). An APC's history of antigenic exposure also influences its phenotype (e.g., Johnston *et al.*, 1996).

Antigen presenting cell - $CD4^+$ T-cell interactions

The interaction between MHC class II-bound antigenic peptides on the surface of antigen presenting cells (APCs), and the T-cell receptor (TCR) and CD4 on the surface of T-helper cells is crucial to the initiation of most antigen specific immune responses (Janeway and Travers, 1996). In addition to TCR interaction with antigen-MHC II on the APC cell surface, the T-cell must receive co-stimulatory signals. If the TCR is triggered in the absence of co-stimulation T-cells become anergic or undergo apoptosis mediated through Fas / CD95-Fas-ligand interactions (Wesselborg *et al.*, 1993; Boehme *et al.*, 1995; TucekSzabo *et al.*, 1996; Arimilli *et al.*, 1996; Alberolalla *et al.*, 1997; Chung *et al.*, 1997; Wong *et al.*, 1997; Maier and Greene, 1998; Walker *et al.*, 1998). Th1 cells are reported to be more sensitive to apoptosis via this type of activation than Th2 cells due to their greater Fas-ligand expression (Ramsdell *et al.*, 1994; Zhang *et al.*, 1997; Alberolalla *et al.*, 1997; Oberg *et al.*, 1997; Ledru *et al.*, 1998). Similarly if CD4 alone is cross-linked on the T-cell surface either *in vitro* (Desbarats *et al.*, 1996; Hashimoto *et al.*, 1997) or *in vivo* (Howie *et al.*, 1994; Malcomson *et al.*, 1997) death of the cell results. There are a variety of co-stimulatory signals requiring cell-cell contact including ICAM-1 / LFA-1, CD40 / CD40 ligand and CD28 / B7 all of which trigger intracellular activation pathways in the T-cell (June *et al.*, 1994; Durie *et al.*, 1994; Croft and Dubey, 1997; Chambers and Allison, 1997). Naive $CD4^+$ T-cells have a greater co-stimulatory signal requirement than memory $CD4^+$ T-cells (Wingren *et al.*, 1995; Dubey and Croft, 1996; Carter *et al.*, 1998). In addition to these direct contact interactions, the APC and other local tissue cells (e.g. other leukocytes, stromal cells, epithelial cells, endothelial cells and fibroblasts) release cytokines and other soluble mediators, which also stimulate intracellular activation pathways in the T-cell (Chatila *et al.*, 1987; Vink *et al.*, 1990; McKay and Leigh, 1991; Murphy *et al.*, 1994; Filler *et al.*,

1996; Rochester *et al.*, 1996; Egan *et al.*, 1996; Hilkens *et al.*, 1996; Rasmussen *et al.*, 1997; Joseph *et al.*, 1998). Macrophages, DCs and LCs all process and present antigenic peptides in cell surface MHC II molecules (Hirschberg *et al.*, 1982; Cohen and Kaplan, 1983; Ashwell *et al.*, 1984; Krieger *et al.*, 1985; Kapsenberg *et al.*, 1986; Tiegs *et al.*, 1990; Ellis *et al.*, 1991). APCs with which T-cells interact can differ in their cell-surface co-stimulatory molecule expression. The tissue microenvironment, by determining the soluble mediator milieu, will also influence signals received by T-cells. The overall balance of signals determines whether a naive $CD4^+$ T-cell differentiates into a Type 1 or a Type 2 helper cell or whether a memory $CD4^+$ T-cell becomes functional.

The functional ability of an APC (in common with many other cell types) depends on the cell's lineage, history and its *in vivo* or *in vitro* microenvironment (Winzler *et al.*, 1997; Soares and Finn, 1998; Luft *et al.*, 1998). Naive $CD4^+$ T-cells are preferentially activated by mature DCs compared to macrophages and B lymphocytes, whilst memory T-cells can be activated by all three cell types (Croft, 1994; Dubey and Croft, 1996). Functional competence of APCs can change over time. For example, LCs can phagocytose antigen but lack significant co-stimulatory activity. When activated by uptake of antigen they migrate from the skin and travel in the blood as veiled cells to the T-cell areas of lymph nodes where they once more change their phenotype becoming functional DCs. Once in the lymph node they up-regulate accessory molecules and cytokines and lose their phagocytic properties (VanWilsem *et al.*, 1994). Thus, during the maturation of a DC its functional role changes from that of acquiring antigen, to transporting antigen, to stimulating T-cells (Balfour *et al.*, 1981; Streilein *et al.*, 1990; Cumberbatch *et al.*, 1991; VanWilsem *et al.*, 1994; Rattis *et al.*, 1996; Udey, 1997; Davis *et al.*, 1997).

APC – HIV-1 interactions

HIV-1 interacts with cells of the immune system in many different ways. Many of the effects of HIV-1 have been principally investigated using T-cells or T-cell lines, but it has become obvious that the interaction of HIV-1 with cells extends much further than simply infecting $CD4^+$ T-cells. HIV-1 is able to target a wide range of

cell types for productive infection (see Levy, 1994, for a comprehensive list) which is a multistage process with possibilities for dysregulation of the immune system at every step (Hewson *et al.*, 1999). In addition HIV-1 may disrupt the function of, or even kill, other immune cells without infecting them. Extracellular gp120, present in the serum of HIV-1$^+$ patients (Oh *et al.*, 1992), can also alter the function of APCs.

HIV-1 as antigen

Infected individuals develop CD8$^+$ cytotoxic T-cells which recognise epitopes on a number of HIV-1 proteins, including gp120, and these may control initial infection (Klein *et al.*, 1998; Goh *et al.*, 1999). The surface glycoprotein gp120, probably because of its exposed position on the virion surface, evokes an especially strong antibody response (Spicer *et al.*, 1999). However, the anti-gp120 antibody response is ultimately ineffective in controlling HIV-1 infection in most patients (Wyatt *et al.*, 1998a; Wyatt and Sodroski, 1998b) despite the ability of many anti-gp120 antibodies to block infection *in vitro* (McKnight *et al.*, 1997; Fu *et al.*, 1999). Gp120 is not only found on the virion surface, but can be shed into the extracellular compartment (Oh *et al.*, 1992) and is found in the plasmalemma of infected cells (Blumenthal *et al.*, 1994). In the extracellular compartment gp120 can act as a T-cell (Laurence *et al.*, 1992) and B-cell (Karray and Zouali, 1997) superantigen, and cause the functional loss of lymphocyte subsets. The anti-gp120 antibody response of most patients is highly skewed. Antibodies using the VH3 gene segment dominate normal human antibody responses. However antibody responses to HIV-1 in infected individuals rapidly lose any contribution from VH3 and there is over-representation of the VH4 locus. The deletion of VH3-using B-cells is attributed to a gp120 superantigen incorporating sites in the C2 and V4C4 domains of gp120 (Karray and Zouali, 1997). The exception to this is seen in long-term non-progressors who do have VH3 antibodies present in their serum but do not make antibodies to the superantigen determinant (Jones *et al.*, 1998).

Gp120 induces changes in APC cytokine production

Gp120 in the absence of any other viral component is able to induce interferon (IFN, mainly α with some γ) production in PBMCs (Ankel *et al.*, 1996). The inductive effect of gp120 is abrogated by the addition of soluble CD4 (sCD4) and is dependent on the V3 loop. This suggests that binding and presumably the resultant clustering of CD4 and a chemokine receptor is required and sufficient for IFN production to result.

More recent work (Gessani *et al.*, 1997) has shown that the ability of APCs to produce cytokines in response to gp120, and the ability of cells to respond to cytokines can depend on the differentiation state of the cell. As monocytes differentiate to macrophages they show an enhanced IFN-β production in response to HIV-1 infection or LPS (bacterial lipopolysaccharide) or gp120 treatment (Gessani *et al.*, 1997). Concomitant to this, the cells' sensitivity to IFN, as measured by the induction of protection from VSV (vesicular stomatitis virus) granted by IFN-β, increased with differentiation to macrophages because of up-regulation of IFN receptors.

IL-10 secretion in response to gp120 was also observed, but the level of this did not depend on the cells' differentiation state. IL-10 secretion could cause the switch from Th1 to Th2 helper subtypes observed in HIV-1 disease (Clerici and Shearer, 1993). Cells only produced IL-12 in response to gp120 if they had been previously primed by IFN-β. Only macrophages could be primed by IFN-β, presumably due to their greater sensitivity to this cytokine (Gessani *et al.*, 1997).

The effects cytokines have on the rest of the immune system and on HIV-1 replication are complex. IFN is able to down-regulate HIV-1 expression in macrophages (Poli and Fauci, 1993). In contrast, TNF-α has been shown to up-regulate HIV-1 expression in macrophages by most (Rabbi *et al.*, 1997; Fortis *et al.*, 1999), but not all (Lane *et al.*, 1999) researchers. The question of whether TNF-α production is of benefit to either the patient or HIV-1 remains under debate and is complicated by the fact that although TNF-α is generally regarded as increasing HIV-1 production by cells, it can also cause an increased production of infection-blocking cytokines (Lane *et al.*, 1999) and an enhancement of NK and LAK mediated killing of infected cells (Fortis *et al.*, 1999). The exact role of many

cytokines, including the novel chemokines and other newly identified signalling molecules, awaits further investigation.

Does HIV-1 induce a Th1 to Th2 switch?

The Th1 to Th2 switch hypothesis as proposed by Clerici and Shearer (Clerici and Shearer, 1993) states that progression to AIDS is dependant on a switch from Th1 to Th2 as the dominant T-helper subset (figure 1.7). The evidence to support this assertion includes the observation that in short-term PBMC culture, cells taken from patients of increasing clinical progression show a concomitant increase in IL-4 and IL-10 production and a loss of IL-2 and IFN-γ production (Clerici and Shearer, 1993). In addition, data show that T-cell clones from HIV infected skin biopsies are more likely to be classified as Th2 clones than clones derived from healthy control skin (Romagnani *et al.*, 1994a; Romagnani *et al.*, 1994b). Clerici and Shearer show that many HIV-1 exposed but uninfected individuals are able to generate strong Th1 type responses, and suggest that these individuals are protected from disease by the failure to undergo the normal Th1 to Th2 switch (Clerici and Shearer, 1993).

The mechanism by which the switch operates may involve T-cell or APC produced cytokines to bias the Th response to Th2. For example, in PBMCs and lung macrophages the HIV-1 protein Tat inhibits production of the Th1 cytokine IL-12 (Ito *et al.*, 1998).

There is, however, some evidence that argues against the Th1 to Th2 switch hypothesis (Graziosi *et al.*, 1994; Maggi *et al.*, 1994; Miedema *et al.*, 1994). There has been difficulty in reproducing the observations by Clerici and Shearer on IFN-γ, IL-1 and IL-4 production in short term PBMC culture system (Romagnani *et al.*, 1994a). There are arguments against using PBMCs for such experiments because as well as containing Th-cells, PBMCs contain monocytes, B-cells, NK-cells and $CD8^+$ T-cells all of which are capable of producing cytokines. The proportions and absolute numbers of these different cell types change as disease progresses. Changes in the cytokine profile attributed to a Th subtype switch could be due to selective deletion of Th-cell subtypes. The T-cells present in skin biopsies may not reflect the types found elsewhere in the body.

The Th1 to Th2 switch hypothesis has recently become more complicated with evidence that Th2 and Th0 cells are able to replicate HIV-1 more efficiently than Th1 cells (Maggi *et al.*, 1994; Vyakarnam *et al.*, 1995). Whether this translates into a longer or shorter life span for the infected cells is unclear. IL-4 has been shown to up-regulate, and IL-12 down-regulate CXCR-4 expression and therefore infectability by the T-cell tropic HIV-1 strains associated with disease progression (Meyaard *et al.*, 1996; Klein *et al.*, 1997; Suzuki *et al.*, 1999). This observation might argue for a Th2 (IL-4) shift increasing selective pressure on HIV-1 to use CXCR-4 and explain why we see a shift in coreceptor usage from CCR-5 to CXCR-4 as disease progresses (Zhang *et al.*, 1998). However, another Th2 cytokine, IL-10 increases CCR-5 (associated with macrophage tropism) expression (Graziosi *et al.*, 1994; Romagnani *et al.*, 1994a), so the picture is far from clear.

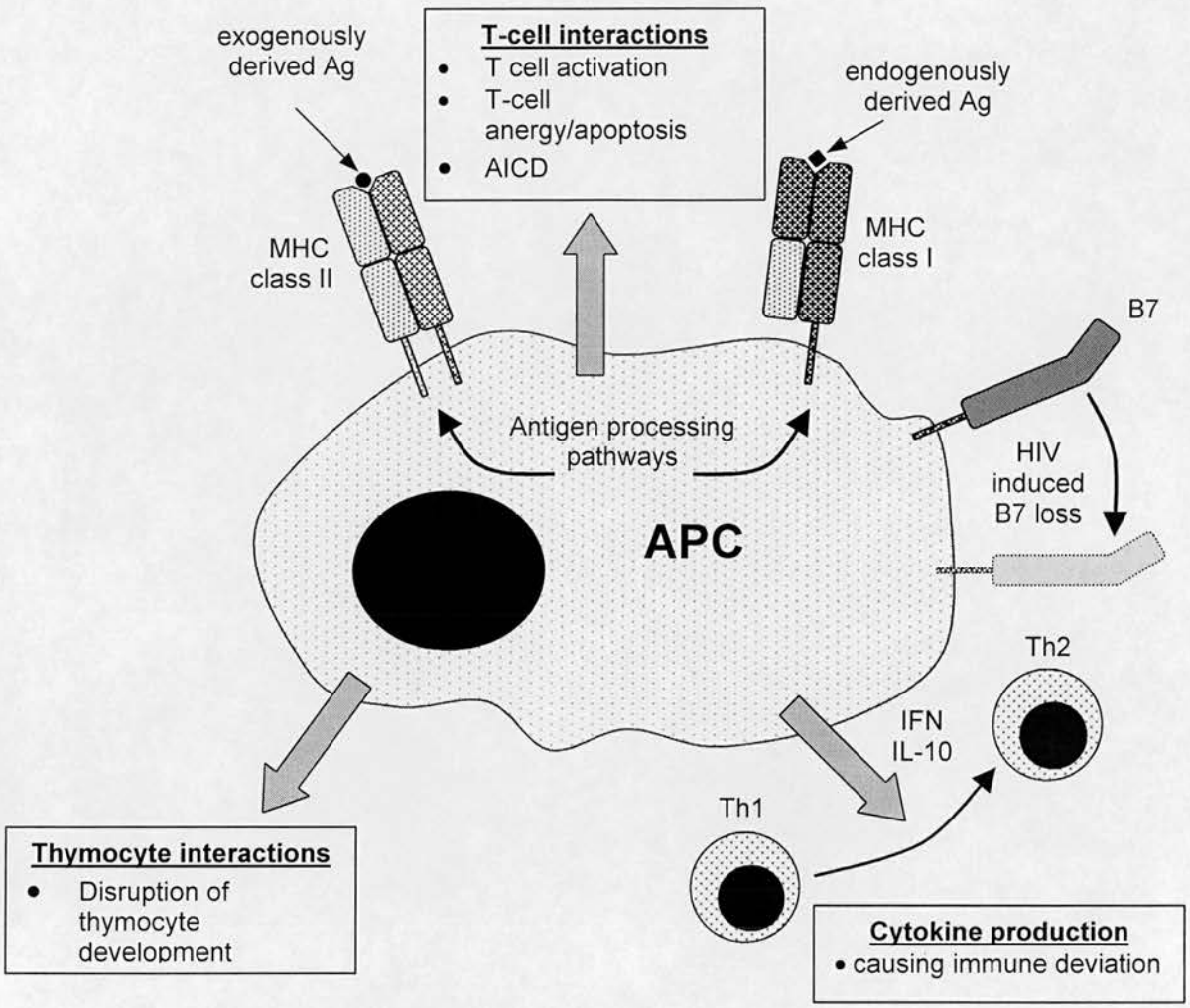


Figure 1.5. HIV-1 control of antigen presentation and APC-T-cell interactions. Anti-HIV-1 T-cell responses are observed in HIV-1 disease so APCs must be able, on occasion, to present HIV-1 protein epitopes together with costimulatory signals to T-cells. However, HIV-1 infection is able to induce B7 (CD80) loss from the surface of APCs; this can result in antigen (from HIV-1 and other pathogens) being presented to T-cells in a context which, rather than activating the T-cell, can induce T-cell anergy or activation induced cell death. As well as giving inappropriate signals to mature T-cells, HIV-1 infected APCs can disrupt thymocyte development by inappropriate intra-thymic signalling. HIV-1 infected APCs can also affect the nature of a T-cell response by producing cytokines such as IFN and IL-10 which cause immune deviation from a Th1 to a Th2 response, the Th2 response being less effective at combating HIV-1 disease.

Figure adapted from Hewson *et al.*, 1999. Artwork by Nazir Lone. © Blackwell Science Asia, 1999.

Gp120 induces a cell-signalling defect

In T-cells gp120 is able to cause dissociation of cell surface CD4 from cytoplasmic $p56^{lck}$ (Hubert *et al.*, 1995). This results in down-regulation of CD4 expression (a possible mechanism of avoiding HIV-1 superinfection) and an abrogation of signalling through the TCR / CD3 complex (Hubert *et al.*, 1995). In the context of macrophages, CD4 plays a different role to that in T-cells (Center *et al.*, 1995) but it may still be associated with a protein kinase, which becomes phosphorylated (activated) as a result of CD4 cross-linking by anti-CD4 antibody or gp120. $P56^{lck}$ has never been reported in primary monocytes or macrophages although other *src* kinases such as Lyn are present (Bowers *et al.*, 1997) and there is a single report of $p56^{lck}$ being found in the monocytic cell line THP-1 and being phosphorylated in response to CD4 cross linking by anti-CD4 or gp120 (Hui *et al.*, 1995). It is also possible that gp120 can inhibit signalling through CD4 in macrophages by causing CD4 / *src* kinase dissociation and reduced CD4 expression.

Gp120 may also interfere more directly with CD4 function by competing with its other ligands (MHC class II on APCs and IL-16). It has also been suggested (Levy, 1994), although not proven, that anti-idiotypic antibody mirroring gp120 may have a role in HIV-1 pathogenesis.

Other HIV-1 proteins induce cell-signalling defects

Nef and Vpu are two other HIV-1 proteins that have been shown to induce cell-signalling defects in infected cells.

Many activities have been attributed to Nef; the function for which it evolved is still not fully understood. It is needed to maintain high viral loads (Kestler *et al.*, 1991) and may achieve this by inhibiting the super-infection of cells (this could result in the death of a cell before it had been used to produce many virions, De *et al.*, 1998) or by optimising protein sorting to the viral membrane during virus particle assembly (Craig *et al.*, 1998).

When the monocyte / macrophage-like cell line, U937, was transfected with *nef* a down regulation of FcγRI and FcγRII, and a changed cytokine response to LPS and PMA were observed (De *et al.*, 1998). In myeloid cells *nef* transfection caused

an up-regulation of MHC class I surface expression, in contrast to T-cells where *nef* transfection induced a down-regulation of MHC class I (Peter, 1998) by causing rapid endocytosis of Nef and MHC class I complexes (Schwartz *et al.*, 1996). Nef has also been demonstrated to induce a CD4 down-regulation, at least in T-cells (Rhee and Marsh, 1994). The HIV-1 protein Vpu is unique among primate Lentiviruses in being only found in HIV-1 and the closely related SIV_{CPZ} (Cullen, 1998), it complexes with nascent CD4 in the endoplasmic reticulum and leads to its retention and degradation (Willey *et al.*, 1992).

The role of gp120 in inducing leukocyte apoptosis

It has been reported (Bour *et al.*, 1995) that gp120 interacting with its receptors in the absence of signalling through the TCR can lead to T-cell anergy and priming of the T-cell for AICD (activation induced cell death, a form of apoptosis) upon receiving a subsequent signal via the TCR. Whether gp120 can prime APCs for apoptosis, and what trigger would result in cell death is not known (figure 1.5).

It has also been reported (Cottrez *et al.*, 1997) that HIV-1 infected APCs can prime HIV-1-specific T-cells to undergo AICD. The priming of cells requires two signals from the APC to the T cell to be delivered simultaneously. The first signal is antigen specific and is delivered through the TCR. The second signal is delivered (presumably via CD4 and/or chemokine receptors) by gp120 expressed on the surface of the APC. Experiments involving the transfection of monocytes to express single HIV-1 proteins have shown that gp160 alone is sufficient to constitute the second signal and that no other viral component is required. The second signal can be blocked, and cells rescued from AICD, by the addition of the CD4 ligand, IL-16 (Idziorek *et al.*, 1998).

AICD can result in hidden damage to the immune system in the absence of a significant decline in overall T-cell number. T-cell clones, that are required to provide protection against HIV-1 and other pathogens present in the host, are selectively destroyed because it is these T-cells which will receive antigen-specific signals from APCs.

It has been reported that HIV-1 infection of macrophages leads to increased expression of Fas ligand (FasL) by macrophages (Badley *et al.*, 1996; Dockrell *et al.*,

1998), and that this mediates apoptosis of uninfected T-cells (Badley *et al.*, 1996). Recent controversy over the reliability of anti-FasL reagents (Restifo, 2000), however, casts doubt on the validity of these results.

HIV-1 infected APCs may be unable to deliver appropriate co-stimulatory signals to $CD4^+$ T-cells (Mosier and Sieburg, 1994); this could result in T-cell anergy or apoptosis. Monocytes / macrophages in HIV-1 disease may produce subnormal levels of IL-12 resulting in T-cell death (Gougeon and Montagnier, 1993b; Ameisen *et al.*, 1994; Ameisen *et al.*, 1995). T-cell deletion in HIV-1 disease may be the result of an HIV-1 encoded super-antigen; although this has yet to be identified, some authors have suggested Nef as a candidate (Montagnier, 1995; DeSimone *et al.*, 1996). There is some evidence (Buttke and Sandstrom, 1994; Greenspan and Aruoma, 1994) that the HIV-1 gene, Tat, can be secreted by infected cells and taken up by surrounding non-infected cells (Frankel and Pabo, 1988; Zauli *et al.*, 1992; Westendorp *et al.*, 1994). Once in cells, Tat can induce oxidative stress by activating NF-κB and TNF-α expression (Dezube *et al.*, 1992); such oxidative stress can prime cells for apoptosis. Additionally, Tat may be able to directly down-regulate the expression of *bcl-2*, an anti-apoptotic gene (Derossi *et al.*, 1994).

Other HIV-1 proteins can be cytotoxic in isolation. Gp41 is toxic to cells probably through increasing membrane permeability (Miller *et al.*, 1993). Domains of Nef, Tat and gp41 all show similarities to neurotoxins (Werner *et al.*, 1991; Garry and Koch, 1992). Nef can change the membrane potential of cells (Werner *et al.*, 1991) and Tat has been demonstrated to cause neurone death (Sabatier *et al.*, 1991).

HIV-1 / cell binding and membrane fusion: potential for dysregulation

As discussed previously, HIV-1 binds to the surface of target cells by using gp120, which interacts with host CD4 and a chemokine receptor. Although both cellular receptors are usually required for infection there have been reports (for example, Livingstone *et al.*, 1996) of HIV-1 infected $CD4^-$ $CD8^+$ T-cells. Possible mechanisms for this include a CD4-independent infection mechanism (Clapham *et al.*, 1996), possibly involving the use of galactosylceramide (Hammache *et al.*, 1996), or the infection of double positive thymocytes. An alternative explanation would be that the $CD8^+$ T-cells became infected during activation, where a transient expression of CD4

is seen (Flamand *et al.*, 1998). This last mechanism would cause preferential loss of the very $CD8^+$ cells needed to protect against pathogens.

The efficiency of HIV-1 infection of APCs and other cell types bearing Fc receptors or complement receptors can be enhanced by non-neutralising antibody, a phenomenon known as antibody-dependent enhancement (ADE, Prohaszka *et al.*, 1997). In some studies (Toth *et al.*, 1994) ADE abrogated the requirement for CD4 binding and allowed the infection of $CD4^-$ cells. In other studies (Perno *et al.*, 1990) CD4 / gp120 binding was reported as essential for infection. Ligation of Fc receptors and complement receptors must also have implications with respect to intracellular signalling, regardless of whether infection results.

In some cases HIV-1 may bind to the surface of a cell in the absence of envelope / plasmalemma fusion and without infection of the cell, possible by DC-SIGN mediated interactions (Geijtenbeek *et al.*, 2000a). This bound virion could 'piggy-back' on a migrating cell and lead to the dissemination of the virus. DCs have been implicated in transporting bound virus from mucosal entry sites to lymph nodes (Spira *et al.*, 1996).

After virion binding, in order for infection to become established, the target plasmalemma must fuse with the viral envelope. Fusion appears to be mediated by a fusogenic portion of gp41 (Pereira *et al.*, 1997). It is important to remember that both the inner and outer lipid monolayers must fuse and that each fusion may be an independent event. It is possible that partial-fusion could result in the core being unable to enter the cytoplasm. Neutralising antibodies mostly act by preventing virion binding (Chamat *et al.*, 1992); some, especially those against gp41, may interfere with the envelope protein's fusogenic function. Some anti-AIDS drugs also work by disrupting plasmalemma / envelope fusion (see chapter 6).

Factors effecting HIV-1 infection and disease progression

Several host factors have been shown to effect HIV disease progression rate (for a review see Roger, 1998). As well as providing clinically useful prognostic markers, an understanding of the mechanisms involved in controlling infection and progression rates may be helpful in the search for novel therapeutic approaches.

Genetic factors so far identified as important include chemokine receptor polymorphism (see below and chapter 3), HLA polymorphism and less clearly defined host factors which contribute to differential levels of cytokine and chemokine production and immune cell activity.

HLA polymorphisms and HIV-1 disease progression

HLA (MHC class I and II) genotype has been shown to influence the time taken for HIV-1 disease to progress to AIDS. Certain alleles or allelic combinations (for example, B27, B51 and (A25+TAP2.3) are protective, whilst other alleles such as B37 and (B6+(TAP2.1 or TAP2.3) are associated with rapid progression (Saah *et al.*, 1998). HLA polymorphism may exert its effects through differences in HIV-1 antigen presentation causing different efficiencies of anti-HIV-1 immune response (Tomiyama *et al.*, 1997). An alternative explanation involves molecular mimicry. The V3 loop of HIV-1 gp120 mimics HLA DR5 and HLA DR6, and this is known to influence the anti-HIV-1 TCR repertoire by the deletion of self reactive $CD8^+$ T-cells during central self-tolerance induction (Itescu *et al.*, 1994).

HLA allele associations with disease progression rates may be due to genetic linkage between HLA loci and other loci of the MHC such as those that code for the TNF and complement components. TNF-α can induce HIV-1 infected cells to produce virus through activation of NF-κB. Polymorphisms of the *tnf-α* promoter have been associated with differential rates of disease progression (Khoo *et al.*, 1997; Brinkman *et al.*, 1997). The complement component, C4, has two null alleles, which are associated with low plasma C4 concentrations, poor antibody responses and rapid disease progression (Cameron *et al.*, 1990; Hentges *et al.*, 1992).

Other possibly important host factors

A Danish study (Garred *et al.*, 1997) showed an association between homozygosity for loci conferring low serum levels of mannose binding protein (MBP) and increased HIV-1 susceptibility and shorter survival time from AIDS diagnosis.

Many cytokines, especially those which are pro-inflammatory (TNF, IL-1β, and IL-6) up-regulate viral replication in infected cells, whilst other cytokines (Il-4,

IL-10 and IFN-β) down-regulate HIV-1 production (Cohen *et al.*, 1997a). It is conceivable that genetic polymorphisms in the inducibility of such cytokines could influence HIV-1 disease progression rate.

β-chemokines inhibit macrophage-tropic HIV-1 infection (Zagury *et al.*, 1998; Cota *et al.*, 2000), and α-chemokines such as stromal cell-derived factor (SDF-1) inhibit T-cell-tropic HIV-1 infection (Oberlin *et al.*, 1996). A G to A substitution in the promoter of *sdf-1* has been shown to accelerate progression to AIDS (Winkler *et al.*, 1998; VanRij *et al.*, 1998).

Virus genetic factors effecting HIV-1 disease progression

The lack of HIV-1 disease progression in some long-term survivors (LTSs) can not be attributed to any as yet identified host factors. An alternative explanation for the lack of disease progression is that at least some of the LTSs are infected with HIV-1 of a low pathogenicity (Huang *et al.*, 1998). Rapid clinical progression is associated with rapid viral replication (Schuitemaker *et al.*, 1992; Connor and Ho, 1994). Defects in the viral genes *nef, vif, vpr, vpu, tat, rev, gag* and *env* have all been associated with slowed replication or delayed clinical progression. (Kirchhoff *et al.*, 1995; Deacon *et al.*, 1995; Michael *et al.*, 1995a; Michael *et al.*, 1995b; Premkumar *et al.*, 1996; Mariani *et al.*, 1996; Wang *et al.*, 1996; Zhang *et al.*, 1997; Menzo *et al.*, 1998; Huang *et al.*, 1998). A methionine to isoleucine substitution in the initial amino-acyl residue of Gag and premature stop codons have been associated with long-term survival (Huang *et al.*, 1998). A G to A nucleotide substitution in the LTR results in a low viral load and long-term survival (Zhang *et al.*, 1997). A shift from a CCR-5- to a CXCR-4-tropic virus population as controlled by the sequence of *env* is associated with disease progression (Schuitemaker *et al.*, 1992). LTSs have also been identified infected with HIV-1 that carries a rare *env* mutation which renders the virus nearly completely unable to infect $CD4^+$ cell lines, activated PBMC or macrophages (Menzo *et al.*, 1998).

Chemokine receptor polymorphisms and disease progression rate

The study of coreceptor usage has lead to some interesting clinical observations. *Ccr5* and *ccr2* are both closely linked on chromosome 3p21-22 (Martin *et al.*, 1998b). *Ccr5* has three relatively common alleles; wildtype, and two mutations, the *Δ32* deletion (Mummidi *et al.*, 1998) and the *m303* premature truncation (Quillent *et al.*, 1998). The most frequent *ccr5* null mutation is the *Δ32* deletion which will be discussed in detail in chapter 3. Both mutant alleles result in a failure of functional receptor to appear on the cell surface. In populations of European descent, homozygosity for a null mutation in the *ccr5* gene is present at surprisingly high frequencies; about 18% are heterozygous for a *ccr5* mutation and about 1% are homozygous (see chapter 3 and Landau, 1997).

The mutations do not confer a selective disadvantage on healthy individuals but, at least when homozygous, protect the individual against infection by HIV-1 or confer a long-term non-progressive disease course (Michael *et al.*, 1997). The reasons why such individuals do not become infected by virus utilising an alternative coreceptor (X4 / T-tropic strains using CXCR-4 for example) are obscure. It could be that M-tropic strains (using CCR-5) are responsible for the initial mucosal infection of APCs required for sexual transmission of HIV-1 (Landau, 1997). Mucosal epithelia constitutively express the CXC chemokine SDF-1; this causes down-modulation of CXCR-4 on mucosal and submucosal lymphocytes (Agace *et al.*, 2000). This may explain why transmission across mucosal surfaces is rarely mediated by infection of T-cells with X4-tropic HIV-1 strains.

During asymptomatic HIV-1 infection, the virus replicates rapidly and with low fidelity (Ho *et al.*, 1995; Grossman *et al.*, 1999). This produces a great deal of diversity in viral proteins and can allow HIV-1 to evolve resistance to therapeutic drugs. However, during the asymptomatic phase it is usually only possible to isolate CCR-5 utilising virus. It is only during the symptomatic phase, when the immune system collapses, that broadening of coreceptor usage is seen (Schuitemaker *et al.*, 1992). There is also a switch from the non-syncytium-inducing (NSI) to the more cytopathic syncytium-inducing (SI) viral phenotype (Connor and Ho, 1994). It has been suggested (Landau, 1997) that in the early-stages of infection the immune

system suppresses viruses with expanded coreceptor specificity, although the mechanism by which this would be achieved remains obscure.

The extent of protection from infection and disease progression gained by the *ccr5 wt/Δ32* genotype is controversial. Protection is probably only partial and may only be from transmission by heterosexual sex and not from homosexual and intervenous infection routes (Hoffman *et al.*, 1997; Edelstein *et al.*, 1997; Rousseau *et al.*, 1997; Roger, 1998). A *ccr2b* mutation, *64I* (valine to isoleucine substitution) has also been epidemiologically linked to reduced disease progression rate in HIV-1^+ individuals (Kostrikis *et al.*, 1998; Roger, 1998; EugenOlsen *et al.*, 1998; Magierowska *et al.*, 1999). The amino-acyl residue substitution manifested in the *64I* allele is conservative and found in a trans-membrane region of the protein. This observation, together with the fact that HIV-1 rarely uses CCR-2b as an important coreceptor (Zhang *et al.*, 1998), suggests that the *ccr2b64I* allele may not effect disease progression directly. It is possible that the *ccr2b64I* allele is a linkage marker for another locus that is able to confer protection. One candidate locus to show a linkage to *ccr2b* is the *ccr5* promoter (*ccr5p*). An A/G polymorphism at the *ccr5p* locus has been linked to lower promoter activity and a progression to AIDS three or four years more slowly than the wildtype *ccr5p* (McDermott *et al.*, 1998; Garred, 1998). Alternatively, the *ccr2b64I* allele may influence chemokine secretion or CCR-5 or CXCR-4 expression (Horuk, 1999). In the absence of any demonstrated effect of the *ccr2b64I* allele on *ccr5* mRNA levels (Mariani *et al.*, 1999), it has recently been suggested that the mutant form of CCR-2b is able to form a heterodimer with wildtype CCR-5 and sequester this in a form unusable to HIV-1 (Mellado *et al.*, 1999).

HIV-1 replicative cycle

After fusion with a cell, the core of the HIV-1 particle enters the host cytoplasm, and the genome and reverse transcriptase molecules are unpacked. The single-stranded RNA genome is reverse transcribed and eventually forms double-stranded DNA. This DNA is then transported to the cell nucleus where it is circularised and integrated into a random site on the host DNA (Varmus, 1988). Non-integrated viral

DNA may be able to produce infectious HIV-1, but integration is a requirement for efficient, long-term virion production (Stevenson *et al.*, 1990).

HIV-1 can enter resting $CD4^+$ T-cells, and the initiation of reverse transcription can take place in these cells (Sun *et al.*, 1997). Formation of the full length viral DNA requires the cell to be activated by TCR ligation (signal one), a signal which normally regulates the G_0 to G_1 transition. Transport of the viral DNA to the nucleus of T-cells to allow for integration requires an additional signal (signal two, co-stimulation via CD28 ligation). The second signal was shown to be IL-2 receptor dependent and sensitive to cyclosporin A (Sun *et al.*, 1997). The signals required to stimulate HIV-1 production in APCs may be different from those required by T-cells; but the differentiation / activation state of the cell will still be important (figure 1.6). For discussion of the dynamics of HIV-1 replication, see (Zack *et al.*, 1990; Zack *et al.*, 1992; Finzi and Siliciano, 1998; Grossman *et al.*, 1999)

Although macrophages express less CD4 than their monocyte precursors, macrophages are more susceptible than monocytes to HIV-1 infection (Valentin *et al.*, 1991). In contrast to resting T-cells, non-dividing, resting macrophages can become productively infected (Weinberg *et al.*, 1991). However, macrophages produce HIV-1 at a slower rate than do T-cells. This may be a result of smaller intracellular pools of nucleotides and other precursors in macrophages, which have slower division rates than T-cells.

GM-CSF has been implicated in some of the signals controlling HIV-1 production by APCs of the monocyte / macrophage lineage. GM-CSF, a cytokine produced by many cell types including activated T-cells, can effect the replication of HIV-1 in cells of macrophage lineage at several levels (Crowe and Lopez, 1997; Manfredi *et al.*, 1997; Kedzierska *et al.*, 1998).

GM-CSF promotes monocyte survival through inhibition of apoptosis and stimulation of proliferation, which increases the number of HIV-1 targets. GM-CSF also promotes the differentiation of monocytes. GM-CSF on its own stimulates differentiation to macrophages; whilst with IL-4 also present, monocytes are driven to become dendritic-like cells (Kiertscher and Roth, 1996; Chapuis *et al.*, 1997). Differentiation of monocytes down either of these pathways appears to result in the

down regulation of surface CD14 (Kruger *et al.*, 1996), although one study (Bagasra *et al.*, 1992) claims that in the monocyte / macrophage cell line U1, GM-CSF up-regulates CD14 expression. GM-CSF-driven differentiation may be important in HIV-1 disease because it turns monocytes, cells that are relatively resistant to infection, into more likely target cells. GM-CSF sources include macrophages / monocytes (Sasaki *et al.*, 1999; Malur *et al.*, 1999); epithelial cells of the nose (Terada *et al.*, 1999), bronchioles (Sanders *et al.*, 1999), colonic mucosa (McCartney *et al.*, 1999), and retina (Crane *et al.*, 1999); nasal endothelial cells (Terada *et al.*, 1999); synoviocytes (Breese *et al.*, 1999); and airway smooth muscle (Hallsworth *et al.*, 1999) and fibroblasts (Spoelstra *et al.*, 1999).

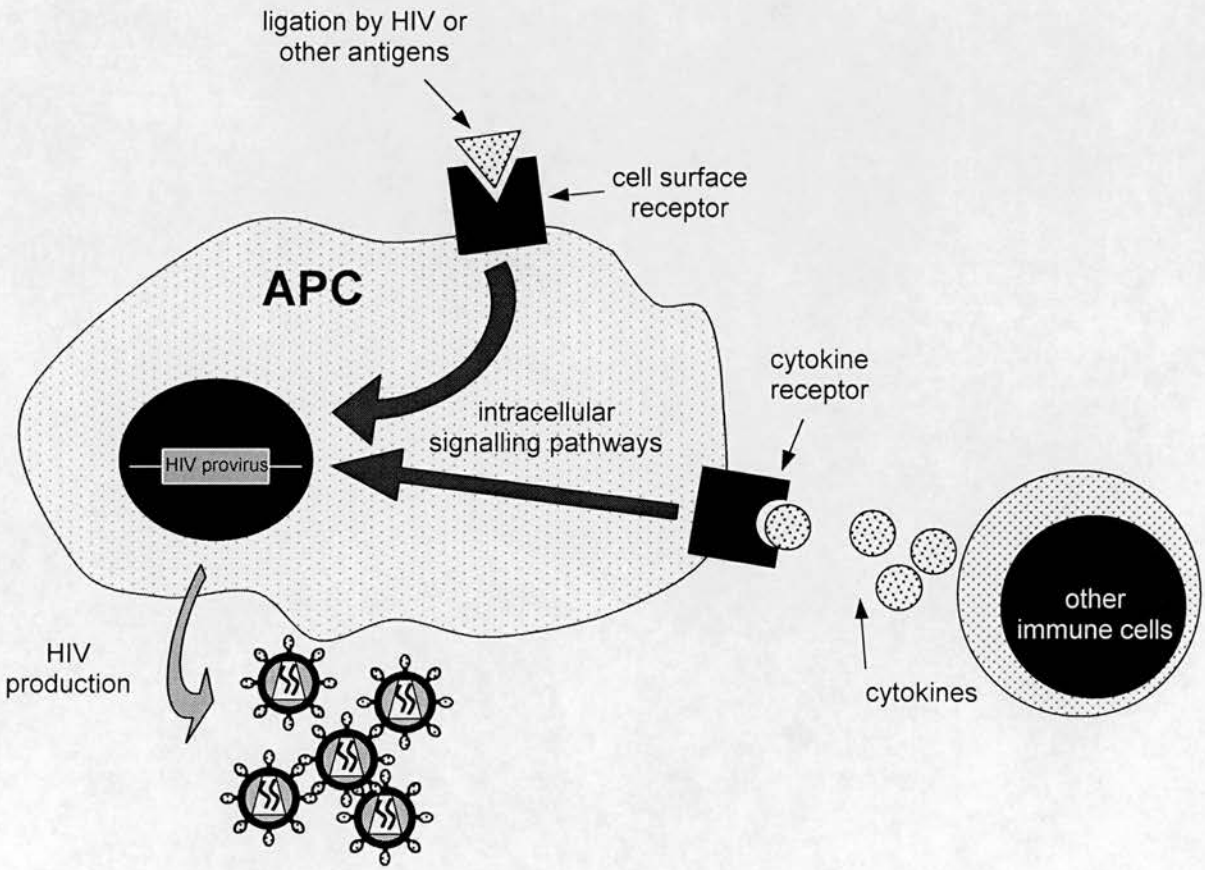


Figure 1.6. Regulation of virus production by APCs. The rate of HIV-1 production by APCs is highly influenced by the activation state of the cell. Signalling through cell surface receptors such as CD4 (see figure 1.4) can effect the cellular activation state. Cytokines produced by other cells in

response to HIV-1 or other pathogens also have an influence on HIV-1 production. IFN and IL-10 have been shown to down regulate HIV-1 production, other cytokines such as TNF-α may stimulate an increase in HIV-1 production. The cell's maturation / differentiation state, as influenced by the tissue microenvironment and cytokines such as GM-CSF, can alter the cell's susceptibility to infection, or the rate of HIV-1 production by an infected cell.

Figure adapted from Hewson *et al.*, 1999. Artwork by Nazir Lone.

The long terminal repeat (LTR): HIV-1's promoter

After HIV-1 DNA has been integrated into a host chromosome, the production of new virions requires the transcription, by host transcriptases, of the provirus to produce both viral mRNA and genomic viral RNA for packaging. Transcription of viral genes is under the control of the LTR, a viral promoter found directly 3' and 5' to the viral genes. The effects of cellular activation and cytokines on HIV-1 expression can be explained in terms of the transcriptional effects on the LTR (Vicenzi *et al.*, 1997; Rabbi *et al.*, 1997; Roebuck and Saifuddin, 1999).

The initiation of HIV-1 transcription is under the control of cellular factors which bind to the LTR. Nuclear factor-κB (NF-κB), nuclear factor of activated T-cells (NFAT), activation protein 1 (AP-1, consisting of a Jun / Fos heterodimer) and Sp-1 have all been shown to activate HIV-1 transcription (for reviews see Gaynor, 1992; Roebuck and Saifuddin, 1999). Once transcription has begun, the viral protein Tat (transactivator) can interact with the *tar* regulatory element and the requirement for cellular transcription factors may be reduced.

The transcription factor NF-κB in resting T-cells is sequestered in an inactive form bound to an inhibitory subunit, I-κB. Activation signals received by the cell through the TCR have the downstream effect of phosphorylating I-κB; this allows NF-κB to be released, bind to, and activate both host and viral genes (Briant *et al.*, 1998). Alcami *et al.*, 1995, showed that NF-κB transcriptional activation is an absolute requirement for HIV-1 transcription and that Tat / *tar* mediated amplification can only occur as a result of earlier NF-κB effects.

CD14 levels can also be important in the control of HIV-1 expression. CD14 acts as a receptor for bacterial lipopolysaccharide (LPS) / LPS binding protein complexes and thus mediates responses to LPS (Wright *et al.*, 1990). CD14, therefore, has a role in mediating LPS induced up-regulation of HIV-1 expression in HIV-1 infected monocytes; anti-CD14 antibodies abrogate this up-regulation (Bagasra *et al.*, 1992; Kedzierska *et al.*, 1998).

As mentioned above GM-CSF has been shown to have an effect on CD14 expression (Kruger *et al.*, 1996) as well as an effect on HIV-1 expression by enhancing *in vitro* virus production in primary monocytes and macrophages, but not in T-cells or LCs (Crowe and Lopez, 1997). See Vicenzi *et al.*, 1997, for a discussion of the role of other cytokines and chemokines in HIV-1 replication.

Work on the U937 monocytic cell line (Thornton *et al.*, 1996), produced evidence that HIV-1 can subvert the normally anti-viral interferon response to reduce its control over viral replication. HIV-1 has a sequence in its regulatory region mimicking the IFN-stimulated response element (ISRE). The ISRE normally activates transcription of anti-viral defence genes in response to the binding of a member of the IFN regulatory factor (IRF) family. Use of IRF-dependent transcription could be of advantage to HIV-1 because IRF proteins are activated on viral infection.

HIV-1 induced syncytia: implications for HIV-1 transcription

An important *in vivo* and *in vitro* feature of HIV-1 is its ability to induce syncytia (multinucleate cells) by the fusion of an infected cell with other, infected or uninfected, cells (figure 1.7). Cell to cell fusion and syncytia formation involves CD4, a chemokine receptor, gp120 and gp41; it appears to be similar to cell / virion fusion (Levy, 1994). Fusion into syncytia may result from gp160 leaking onto the plasmalemma of infected cells and interacting with CD4 and chemokine receptors on other cells. Usually primary macrophage tropic strains, using CCR-5, do not form syncytia in T-cell lines, whereas viruses able to utilise CXCR-4 often do. The categorisation of strains into syncytium inducing (SI) and non-SI (NSI) phenotypes based on coreceptor usage is, undoubtedly, an over simplification; although

coreceptor usage must be important (Moore *et al.*, 1997) the activity of *nef* is also involved (Horuk, 1999).

It has been suggested (Burke, 1997) that cell-to-cell fusion is an important HIV-1 evolutionary strategy, analogous to sex, which facilitates recombination between viral strains by allowing them to come together in a syncytium, a structure likened to a mating ground. From a molecular-biological point of view, syncytium formation may be important in bringing together transcription factors from different cells to allow efficient HIV-1 expression. It has been suggested (GranelliPiperno *et al.*, 1995) that syncytium formation could allow HIV-1 replication to take place in non-activated memory T-cells. Experiments have shown that NF-κB and Sp-1 are both vital transcription factors for HIV-1. Non-activated T cells fail to express HIV-1 because they contain Sp-1 but not NF-κB. Purified DCs fail to support HIV-1 replication because they lack Sp-1, despite containing high levels of NF-κB. DC / T-cell syncytia bring together the two factors and allow up-regulation of viral transcription in the absence of immune stimulation.

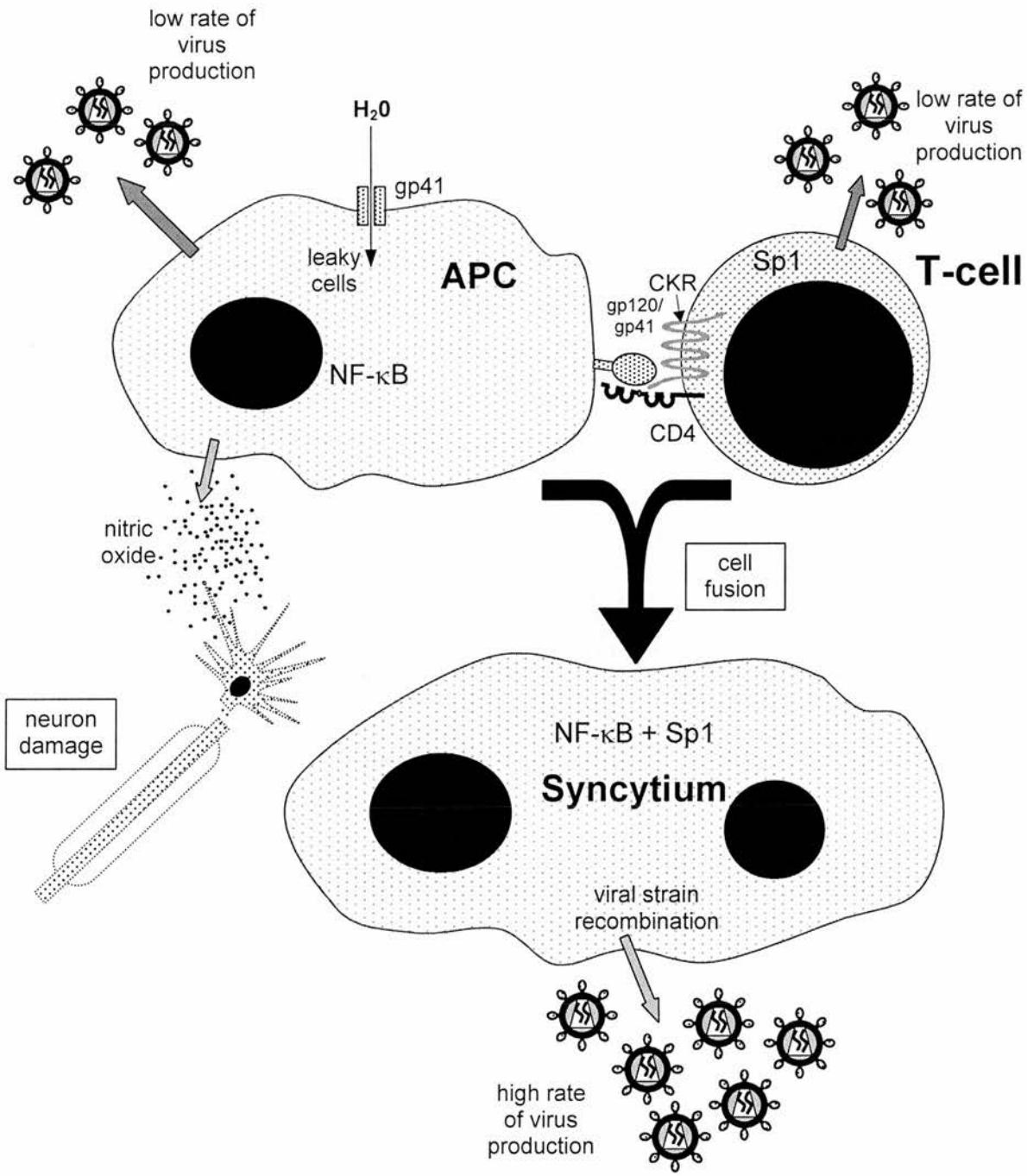


Figure 1.7. Other HIV-1 / APC induced cellular damage. HIV-1 infected macrophages have been shown to produce nitric oxide, which is a neurotoxin. The damage done to neurons in this way may contribute to AIDS-related dementia. Immune system damage may result from the HIV-1 proteins gp41 and gp120 leaking onto the plasmalemma of infected cells. In the case of gp41, this can be directly cytotoxic due to the formation of pores in the cell membrane. Leakage of viral proteins to the cell surface can also

induce cell / cell fusion in a fashion similar to cell / virion fusion. The resulting syncytium not only results in the loss of the daughter cells, but the cell / cell fusion can be advantageous to the virus as it brings nuclear factors important for HIV-1 transcription such as NF-κB and Sp1 together in the same cell; this allows for accelerated virus production. Syncytia may also be sites at which inter-strain recombination can take place, increasing virus genome variability.

Figure adapted from Hewson *et al.*, 1999. Artwork by Nazir Lone.

Immune status and HIV-1 replication rate

The activation state of the host immune system has an important control over HIV-1 replication rates. Vaccination and infection can both activate immune cells, and lead to an increase in viremia (Wahl and Orenstein, 1997). See Rousseau *et al.*, 1999, for a recent review of the responses of HIV-1 infected individuals to a range of vaccinations. Its seems that the stage of HIV-1 disease progression is an important factor when assessing the risks and benefits of offering vaccinations to HIV-1 infected people. Vaccination early in the course of HIV-1 infection and when HAART is being successfully used can elicit beneficial protective immunity to secondary infections. Later vaccination is more likely to promote HIV-1 replication. There is an addition risk associated with the use of live-attenuated vaccines in that the vaccination microbe may be pathogenic in a recipient with an immune system weakened by HIV-1. Increased HIV-1 replication under the influence of other microbes can be a result of changes in immune cell activation or of a more direct interaction. For example, early gene-products of human herpes virus can exert an effect on HIV-1's LTR and enhance HIV-1 expression (Gaynor, 1992).

Recently attention has been given to the therapeutic activation of T-cells with IL-2 under the cover of highly active anti-retrovirus therapy (HAART) in order to flush HIV-1 out of latently infected T-cells (Chun *et al.*, 1999).

Loss of APC numbers in HIV-1 disease

HIV-1 disease leads to a reduction in the number of APCs in the periphery (losses in skin, blood and gut have all been described, Knight and Patterson, 1997b). There are several possible reasons for this loss in cell number (Ng *et al.*, 1996).

The loss of cells may be due to the lysis of infected cells by the CTL (cytotoxic T lymphocyte) response. DCs can be targeted *in vitro* by anti-HIV-1 CTLs (Knight and Patterson, 1997b). It may be that the killing of an APC by the CTL that it has just activated is part of a normal negative-feedback mechanism of controlling excessive T-cell activation. Such a mechanism would only become a problem in HIV-1 disease because of the persistence of infection (leading to sustained, long-term loss of APCs) and the reduced capacity for APC replacement (see below).

Loss of LCs from the skin (Knight *et al.*, 1997a) may simply reflect migration of cells from the periphery to the lymph nodes in response to activation. APCs may be lost by being fused with T-cells during syncytia formation or they may die *in situ* by apoptosis as a direct result of infection. Alternatively a reduction in LC numbers in skin could be due to a failure of haematopoiesis or a failure of tissue colonisation by cells from bone marrow progenitors. $CD34^+$ bone marrow derived stem cells show little capacity to develop (morphologically or functionally) into DCs in patients with advanced AIDS (Knight and Patterson, 1997b).

Levy (1994) suggests that host cells could be destroyed in an autoimmune fashion by other immunocytes which recognise host proteins on these cells as foreign because they have previously been seen linked to an HIV-1 protein (gp120, for example) acting as a hapten. Antibodies to cellular proteins including CD4 and a wide range of autoimmune disorders have been detected in HIV-1 infection (Chams *et al.*, 1988; Schattner and Ragerzisman, 1990; Caporossi *et al.*, 1998). It is possible that loss of APCs is due to targeting by autoimmune mechanisms.

Damage done to the immune system by APCs in the presence of HIV-1

As has been previously noted, HIV-1 infected APCs or APCs that have interacted with HIV-1 proteins show a reduced capacity to stimulate T-cell effector function

(Roberts *et al.*, 1994), and may even prime T-cells for AICD (Banda *et al.*, 1992). The decline in stimulatory capacity may result from the loss of immunologically important APC surface molecules in HIV-1 infection. Gabrilovich *et al.*, 1994, showed that infected DCs down-regulate MHC class II, CD44 and CD54.

APCs are not only important in initiating an immune response, but in determining the direction which the response takes. It has been suggested (Kuchroo *et al.*, 1995) that the divergence of the T-cell response to the Th1 (cytotoxic) or Th2 (humoral) subtype is controlled by differential expression of B7.1 (CD80) and B7.2 (CD86) by APCs. A switch from a Th1 (IL-2 and IFN-γ mediated) to a Th2 (IL-4 and IL-10 mediated) response has been suggested as a critical step in HIV-1-disease establishment and progression. This switch is absent in many seronegative HIV-1-exposed ('resistant') individuals who continue to generate Th1 type responses to HIV-1 (Clerici and Shearer, 1993).

APC type and the concentration and type of antigen may be important in fixing the response type (Knight and Patterson, 1997b). It would be interesting to know to what extent HIV-1 is able to instruct the APC to take a Th1 to Th2 switch (caused by changing expression of B7.1/B7.2 or other surface molecules or by changes in APC cytokine production).

APCs not only exert an effect on T-cells during the induction of an immune response. They are important regulators of T-cell development (figure 1.7). Infected DCs in the thymus may result in inappropriate signalling to developing thymocytes; this would result in the development of an abnormal T-cell repertoire (Knight and Patterson, 1997b). Thymus volume declines during disease progression (Vigano *et al.*, 1999; Vigano *et al.*, 2000)

In her controversial danger hypothesis, Matzinger (1994) suggests a mechanism whereby LCs in the skin can induce tolerance (i.e., anergy or death) in $CD4^+$ T-cells. Her idea is that LCs in the skin express self-antigen / MHC class II complexes but not co-stimulatory molecules. T-cells interacting with these LCs would receive signal one but not signal two and would be tolerised. Matzinger suggests that tissue LCs might phagocytose and present environmental antigen, but viral antigen might also be presented by LCs (at the site of initial infection) to virus

specific $CD4^+$ T-cells in the absence of signal two, this would result in the deletion or anergy of these T-cells.

Of course, it is not only cells of the immune system that are damaged by HIV-1. HIV-1 infection induces macrophages to produce nitric oxide (Bukrinsky *et al.*, 1995), a molecule implicated in the neurological disease seen in some AIDS patients (figure 1.7).

Role of APCs in HIV-1 dissemination

After sexual transmission, HIV-1 is initially localised to the point of entry, but AIDS is a systemic disease with HIV-1 infecting cells throughout the body. APCs have been implicated in allowing virus to spread throughout the body.

SIV infection in rhesus macaques has been used as a model for the early events in heterosexual HIV-1 infection (Miller *et al.*, 1989; Spira *et al.*, 1996). When macaques are inoculated intravaginally with SIV, the virus first appears in, or bound to, DCs of the lamina propria of the vaginal mucosa. Within two days, infected cells are detectable in the draining lymph nodes and by day five, the infection becomes systemic with SIV detectable in the blood (Spira *et al.*, 1996).

DCs can bind HIV-1 and once these cells have matured and are expressing co-stimulatory molecules (this would be in the lymph node *in vivo*) they can stimulate T-cells (Hubert *et al.*, 1995). The stimulation of the T-cells activates them and allows them to support productive infection by the virus passed from the infected DCs. The recently described DC-SIGN integrin (Geijtenbeek *et al.*, 1999; Steinman, 2000; Geijtenbeek *et al.*, 2000a; Geijtenbeek *et al.*, 2000b) provides a mechanism for HIV-1 to piggy-back onto DCs and be transported by them without necessarily infecting them.

Conclusion

It has been 19 years since AIDS was first identified. About 40 million people are now infected with HIV-1 (1% of the world's sexually active population, WHO 1999

figures). There have been some recent advances in treatment (for review see Clumeck and Hermans, 1996) which have allowed the life span of HIV-1^+ patients who can afford these new therapies to be increased. Most of the progress in drug treatment has come from an increased understanding of HIV-1 virology and biochemistry. However, in order to find improved treatments and eventually a cure, and to start to think about reconstructing a battered immune system after successful treatment to reduce viral load, a greater understanding of immunology will be required.

The APC plays a central role in the immune system regulating the actions of other cell types. The goal of HIV vaccine development may also be served by a greater understanding of APC interactions with the viral components of a putative vaccine. A successful outcome of vaccination (the only option for counties that can't afford HAART treatment) depends on appropriate APC / HIV interactions as the first stage of a protective immune response, rather than APC / HIV interactions acting to cause immune system dysfunction and disruption.

An understanding of APC - HIV-1 interactions may hold the key to many of the remaining mysteries of AIDS.

Aims of project

The work described in this thesis was undertaken in order to investigate some of the interactions of HIV-1 with antigen presenting cells. The work aimed to uncover some of the fundamental processes of APC immunology, such as the role of APCs in T-cell activation verus tolerance induction, by learning how HIV-1 is able to subvert them.

Work was particularly focused on the HIV-1 envelope glycoprotein, gp120, and examinations of how this protein is able to dysregulate APCs through interactions with cell surface receptors (CD4 and chemokine receptors). It was also an aim to evaluate potential therapeutic agents and vaccine candidates with a mode of action based on blocking gp120 / APC interactions.

Specific questions

- **CHAPTER 3.** What is the incidence of the *ccr5Δ32* allele in the east of Scotland? How does the allele frequency fit with world-wide distribution patterns?

- **CHAPTER 4.** How does gp120 alter the cell-surface phenotype of APCs? Does gp120 receptor tropism or APC chemokine receptor genotype make any difference to any changes?

- **CHAPTER 5.** Do cellular changes induced by gp120 have any functional consequences for the APC?

- **CHAPTER 5.** Can gp120-induced changes to APCs be explained as a subversion of physiological processes of tolerance induction?

- **CHAPTER 6.** Can potential pharmaceutical agents aimed at preventing HIV-1 from infecting cells by disrupting gp120 / APC interactions be assayed *in vitro* for potential activity?

CHAPTER 2

MATERIALS AND METHODS

Unless otherwise stated, all chemicals were bought from Sigma Ltd (Poole, Dorset, UK) or Fishers Scientific Ltd (Loughborough, Leicestershire, UK). All cell culture reagents and plasticware were bought from Life Technologies Ltd (Paisley, Strathclyde, UK).
See appendix 1 for recipes of solutions described.

Cell separation and tissue culture

Sterile technique was used for all cell preparation and tissue culture steps.

Source of material

Anonymous single-donor Buffy coats were obtained from the Scottish National Blood Transfusion Service (SNBTS). These were made from single unit (450ml) blood donations from individuals giving blood on the previous day in the East of Scotland region. Occasionally, whole blood was taken from healthy laboratory colleagues. In both cases, blood was drawn into containers with anticoagulant present (1 unit per ml of porcine heparin in the case of blood drawn within the Pathology and Respiratory Medicine Departments). Blood collected without anticoagulant for the production of human AB serum was also obtained from the SNBTS.

All blood donors to SNBTS were screened to exclude donors at high risk of carrying blood-borne diseases. Male homosexuals, sex-workers, intravenous drug users and those returning from high-risk countries are among those excluded. Blood donations were screened by the SNBTS for antibodies against HIV-1, HIV-2, hepatitis B, hepatitis C and syphilis. Only blood which was negative in these tests was used. Other blood borne organisms such as cytomegalovirus (CMV) and Epstein-Barr virus (EBV) may have been present in the blood donations used for this work.

Precautions for the safe handling of potentially infective material

Blood from the SNBTS was assumed to be of low infection risk. However, good laboratory practice and 'Universal Precautions' (Kibber, 1997) were adopted. All laboratory personnel working with human material were vaccinated against hepatitis B. Work with viable HIV-1 was carried out in the category-three pathogen-containment facility at the University of Edinburgh Centre for HIV Research. Training and supervision in accordance with the Centre's local rules and code of practice was undertaken.

Preparation of 'off the clot' human serum

Units of whole group AB blood were collected by the SNBTS without an anticoagulant. This blood arrived as a large clot in a bag surrounded by serum. One of the outlet tubes to the bag was surface-sterilised with 70% ethanol and cut. The serum (about 300ml in total) was squeezed into 50ml collection tubes. The serum was centrifuged at 300*g* for 10 minutes to pellet any remaining cells and small clots. Additional centrifugation was carried out, if required, until the serum had attained clarity. The resultant serum was then heated in a 56°C water-bath for 1 hour to inactivate complement. After a final 300*g* / 10 minute centrifugation the serum supernatant was 0.2μm filter sterilised, aliquoted, and stored at -20°C until required.

Isolation of peripheral blood mononuclear cells (PBMCs)

Based on method of Valentin *et al.*, 1991. Mononuclear cells (PBMCs) were isolated from single-donor Buffy coats or whole blood by centrifugation over Lymphoprep™ (Nycomed Pharma AS, Oslo, Norway). Buffy coat or whole blood volume was made up to 200ml with PBS. 25ml aliquots of this blood were then carefully layered onto 15ml of Lymphoprep™ in eight 50ml centrifuge tubes. The tubes were spun at 1000*g* for 18 minutes with no centrifuge brake. After centrifugation the erythrocytes and granulocytes had sedimented and the PBMCs were sucked out of their position at the Lymphoprep™-serum interface using pastettes. The PBMCs were washed in PBS,

centrifuged for 10 minutes at 300*g* and resuspended in 100ml of fresh PBS. The PBMCs were then further purified by a second centrifugation over four tubes of Lymphoprep™ and a second PBS wash. The two stage Lymphoprep™ procedure resulted in PMBCs completely free of red cell contamination and with much reduced levels of platelet contamination. A small sample (about 10^6 cells) of PBMC was retained from each Buffy coat for genotyping purposes.

Cryo-preservation of cells

Cell lines, PBMCs or fractions thereof were frozen for future use by suspending washed cells at about 5×10^7 ml^{-1} in 'freezing mix' (90% FCS, 10% DMSO). Cells were then aliquoted into 1.5ml cryo-tubes. Cells were frozen slowly by wrapping them in paper tissue and placing them in an expanded polystyrene box in a -70°C freezer for at least 2 days. For long term storage, cells were transferred to liquid nitrogen. Cells were thawed at room temperature and the outside of the cryo-tube was sterilised with 70% ethanol. The defrosted cells were washed several times in PBS or medium to remove residues of DMSO. Viable cell recovery was usually about 70%. If required, dead cells were removed by centrifuging the cells over Lymphoprep™ in a method similar to that described above for the isolation of PBMCs.

Macrophage culture

See Bennett and Breit, 1994, for a discussion of the variables involved in macrophage isolation and culture of relevance to HIV research. PBMCs were plated onto tissue culture plates or placed into tissue culture flasks placed on their sides at 5×10^6 cells ml^{-1} in serum-free IMDM containing antibiotics (50 IU ml^{-1} penicillin and 50μg ml^{-1} streptomycin). Monocytes were allowed to adhere to the tissue culture plastic for one hour in a 5% CO_2 humidified 37°C incubator. Non-adherent cells were removed and retained for T-cell culture if required. Adherent monocytes were washed several times with PBS and fresh medium (IMDM + antibiotics as above + 5% heat-inactivated AB normal human serum, SNBTS) was added. Cells were

cultured overnight at 37°C with 5% CO_2 and humidity. The following day floating cells were removed. Adherent cells were cultured for a further 4 to 7 days before use. At this time they were typically >95% $CD14^+$, $MHCII^+$ and $CD4^+$ macrophages by flow cytometry.

Dendritic and Langerhans cell differentiation and culture

Based on methods of Kiertscher and Roth, 1996; Jonuleit *et al.*, 1996; Chapuis *et al.*, 1997; Buelens *et al.*, 1997; Palucka *et al.*, 1998; Woodhead *et al.*, 1998. The majority of human B-cells from most donors are EBV^+ (Ferres *et al.*, 1995; Fathalla *et al.*, 1996). EBV can transform B-cells and occasionally, in culture, a B-lymphoblast clone can proliferate out of control and take over the cell culture. Because IL-4 is a B-cell growth factor (Howard *et al.*, 1983; Farrar *et al.*, 1983) and the dendritic cell differentiation method described here uses IL-4 supplemented medium, dendritic cell cultures are more prone than macrophage cultures to contamination by an EBV^+ B-lymphoblast. Thorough washing of lymphocytes when setting up dendritic cell cultures reduces the chance of B-lymphoblast contamination. All dendritic cell cultures were regularly checked for contamination and any showing blasting lymphocytes were discarded.

Dendritic cells were differentiated from monocytes. Starting with PBMCs the method for macrophage culture as detailed above was followed with the addition of recombinant IL-4 (250 U ml^{-1}, R&D Systems Europe Ltd., Abingdon, Oxfordshire, UK) and GM-CSF (500 U ml^{-1}, R&D Systems Europe Ltd.) to the first serum-containing medium. On day 3, cells were fed with an equal volume of fresh additional cytokine-containing medium. Cells were used on day 7. A Langerhans-like cell type differentiated on the addition of 50 U ml^{-1} of TNF-α (NIBSC Centralised Facility for AIDS Reagents, South Mimms, Hertfordshire, UK) one day before use of the cells (Hausser *et al.*, 1997; Ebner *et al.*, 1998; Steinbach *et al.*, 1998).

$CD4^+$ T-cell purification

Using commercially available columns (Human T-cell CD4 subset column kit, R&D Systems Europe Ltd.) this method was used to purify $CD4^+$ T-cells from PBMC. The manufacturer's instructions were followed and all reagents were provided in the kit. Briefly, a PBMC suspension was incubated with a mixture of monoclonal antibodies and loaded onto the depletion column. The column contained anti-Ig coated glass beads. B-cells and $CD8^+$ T-cells were bound and retained in the column by F(ab) interactions. Monocytes were retained by Fc interactions. The column eluate contained a highly enriched population of $CD4^+$ T-cells.

Gamma irradiation of PBMC

When PBMCs were used as antigen presenting cells in the maintenance of the HA1.7 T-cell clone, they were first gamma-irradiated to prevent proliferation. Up to 2×10^8 PBMCs were suspended in PBS in a 25ml 'Universal' tube and carried on ice to an Atomic Energy of Canada Gammacell 1000 Caesium-137 source (MDS Nordion, Kanata, Ontario, Canada) situated in the blood transfusion laboratories of the Royal Infirmary of Edinburgh. The tube of cells was exposed to the gamma radiation source for 12.8 minutes. This exposure time was calibrated to give the whole sample a dose of >28.8 Gy and no one part a dose of >49.14 Gy. After irradiation, the sample was returned, on ice, to the laboratory.

Maintenance of HA1.7 T-cell clone

The HLA DR1*0101 restricted HA1.7 human T-cell clone (Eckels *et al.*, 1982) was raised against influenza haemagglutinin and shown to react against a synthetic peptide of haemagglutinin ($HA^{306-318}$, Lamb *et al.*, 1982a). It was donated as a growing culture by Prof. Margaret Dallman (Department of Biology, Imperial College of Science, Technology and Medicine, London, UK) along with a supply of $HA^{306-318}$ peptide. It was kept proliferating specifically on a weeklong selection and antigen stimulation cycle. Cells were used for proliferation assays when in their most quiescent state on day 7 before restimulation.

Day 7/0. 1×10^6 T-cells and 1×10^6 irradiated HLA DR1*0101$^+$ PBMCs were mixed in 5ml of RPMI-1640 (+ 2mM L-glutamine + 50 IU ml^{-1} penicillin + 50μg ml^{-1} streptomycin + 5% HI-NABHS) in a 25ml tissue culture flask. 0.5ml of T-LF-Lymphocult® (Biotest (UK) Ltd., Solihull, West Midlands, UK) and 1μg/ml $HA^{306-318}$ peptide was added. Cells were incubated in 5% CO_2 in a humidified 37°C incubator.

Day 3 or 4. Cells were checked. If the medium looked yellow, half the medium was replaced with fresh and 0.5ml of Lymphocult® was added.

Day 7/0. Cells were washed in PBS by centrifugation and either used for a proliferation assay or maintained for another week with fresh PBMCs, medium and peptide (see above).

[^{3}H]-thymidine incorporation proliferation assay

This assay was used to measure the extent of T-cell proliferation. 2×10^5 HA1.7 antigen specific T-cells were placed in 96 well plates in 200μl of IMDM + 5% heat-inactivated AB normal human serum (SNBTS), in the presence of 2×10^4 monocyte derived macrophages or LCs (APCs) from a donor with a presentation competent HLA type.

Cell stimulation. The cells were stimulated by the addition of antigen, or as a positive control, 5μg ml^{-1} of the T-cell mitogen, concanavalin A. Cells were left to proliferate (37°C, humidified, 5% CO_2) for 5 days. 24 hours before the end of the stimulation 1μCi per well of ~35 Ci $mmol^{-1}$ [^{3}H]-thymidine (ICN, Basingstoke, Hampshire, UK) was added. At the end of the cell stimulation period the cells were either harvested immediately or frozen to -20°C to arrest [^{3}H]-thymidine incorporation.

Cell harvesting. A 96 well Tomtec plate harvester (Wallac, Crownhill, Buckinghamshire, UK) was used to harvest the cellular nucleic acids onto a filter mat (Wallac). The mat was dried at room temperature or in a 60°C oven.

Scintillation counting. The filter mat was sealed into a bag (Wallac) with 10ml of 'Betaplate™ Scint' liquid scintillation fluid (Fisons, Loughborough, Leicestershire),

placed into a holding cassette and counted on a 1205 Betaplate™ scintillation counter (Wallac) allowing 20 seconds of counting from each sample.

HIV-1 infection assay

This assay was used to test putative HIV-1-infection blocking agents and to assess the infectability of macrophages of known genotype. Monocyte derived macrophages were set up to culture in 24 well plates as described above. On day 5 the macrophage culture had its medium removed and retained. 100µl per well of the agent under test in PBS (or just PBS as a control) was added to the cells and left for an hour whilst the cells were taken into the category 3 pathogen-containment facility. 150µl of HIV-1_{BAL} supernatant (a gift from Dr Marilyn Moore, University of Edinburgh Centre for HIV Research, Edinburgh, UK) was then added to the cells on top of the existing 100µl of solution. The macrophages were then incubated at 37°C in a humidified 5% CO_2 incubator for 30 minutes. A further 750µl of the original medium was then added to each well. The cells were then returned to the incubator. 72hrs and 96hrs after the addition of HIV-1_{BAL}, RNA was harvested from the cells using Qiagen kits as described below. All RNAs was treated with DNase (see below). Tubes containing RNA were sprayed with 70% ethanol and removed from the category 3 containment lab. The presence of HIV-1 mRNA, and hence, viral entry, reverse-transcription, probably integration, and transcription within the macrophages, was assayed by RT-PCR. 0.1µg of each DNase treated RNA was reverse-transcribed as described below. A HIV-1 mRNA specific fragment of the resultant cDNA was then amplified by semi-quantitative PCR (see below) and the amount of amplified product taken as a measure of the extent of HIV-1_{BAL} infection of the macrophage culture.

Flow cytometry methods

Flow cytometric analysis was carried out using a Coulter EPICS XL flow cytometer (Beckman-Coulter Electronics, Luton, UK) with a 15mW, single argon ion laser operating at λ488nm. Viable cells were gated for analysis using forward and side (90°) scatter characteristics.

Preparation of monoclonal antibodies and control IgG

Cell culture. Hybridoma cell lines secreting antibodies against CD14 (UCHM1) and HLA DR (DA6 231) were obtained, respectively, from Prof. Sir Peter Beverley (The Edward Jenner Institute for Vaccine Research, Compton, Berkshire, UK) and Dr. Keith Guy (Department of Biological Sciences, Napier University, Edinburgh, UK) (Hogg *et al.*, 1984; Gruneberg *et al.*, 1997). Cell lines were growth at 37°C with humidity and 5% CO_2 in about 50ml of RPMI-1640 medium + 2mM L-glutamine + 50 IU ml^{-1} penicillin + 50μg ml^{-1} streptomycin + 10% FCS in a 200ml tissue culture flask. Once a week, most of the supernatant was removed and retained; and about three-quarters of the cells were removed and disposed of. The culture was then made up to 50ml with fresh medium. Retained supernatant was stored at -20°C until approximately 500ml had been collected from each cell line.

Concentrating the supernatant. Thawed supernatant was concentrated by dialysis. About 5ft of 28mm diameter Visking dialysis tubing (Medicell International, London, UK) was wetted with cold tap water and tied securely at one end. Using a funnel, the supernatant was poured into the tubing and the tubing knotted at the open end. The tied dialysis tubing was placed in a plastic box and sprinkled with about 40g of polyethylene glycol. The box was left at 4°C overnight. The following day, the 500ml of supernatant should have reduced in volume to 25-40ml. If further dialysis was required, more polyethylene glycol was added to the tubing, which was then left for a few hours longer. If the supernatant had been over-concentrated, its volume was increased by leaving the tubing in a beaker of distilled water for 20 minutes or so. The tubing was washed free of polyethylene glycol under the cold tap

and the concentrated supernatant removed. The concentrated supernatant was either stored at -20°C or used immediately.

IgG affinity Isolation. This protocol was used to isolate monoclonal IgG from concentrated hybridoma supernatants. It was also used to isolate control, pre-immune serum from mouse and sheep serum (SAPU, Carluke, Lanarkshire, UK). See appendix 1 for details of the solutions mentioned below.

A clamped 7mm × 10cm glass liquid chromatography column (catalogue number C3669, Sigma) was filled with a slurry of 2ml of fast-flow™ protein G conjugated sepharose beads (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) in phosphate buffer (appendix 1). 100ml of phosphate buffer was run through the column to wash the beads free of their ethanol preservative, the flow was stopped just before the buffer reached the top of the beads to prevent them from drying out. About 25-40ml of concentrated hybridoma supernatant or about 3ml of serum was then added to the column and followed by 50ml of phosphate buffer to wash off any unbound proteins. The beads were not allowed to dry out at any stage.

Elution of column. 25ml of glycine / HCl buffer (appendix 1) was run through the column to remove bound IgG. As it came off the column, eluate was collected into tubes in 12 consecutive fractions of about 2ml each. The column was then re-equilibrated with 25ml of phosphate buffer and stored at 4°C with about 2ml of buffer still in the column. 0.02% (w/v) of NaN_3 was added to each column as a preservative, this was washed out before the column was reused.

Selection of fraction(s). 5µl of each fraction was dotted onto a labelled position on an approximately A7 sized piece of nitro-cellulose paper (Amersham Pharmacia Biotech). This paper was dried completely in a 37°C oven and stained in Coomassie blue mixture for 5 seconds. The paper was then destained in destain mixture for 1 minute or until most of the background staining had been removed. The paper was baked at 37°C for 30 minutes between pieces of number 1 filter paper (Whatman, Maidstone, Kent, UK) to intensify the blue staining of the protein dots. Figure 2.1 shows an example of a stained nitro-cellulose paper. The fractions showing the most intense staining (fractions 2 and 3 in figure 2.1) were pooled. All other fractions were discarded. Pooled fractions were adjusted to pH 6.5 – 7.5 by addition of 0.01 M NaOH; pH was tested using indicator sticks (Sigma). Protein content of purified IgG

was measured using the Lowry assay (see below). The purified monoclonal antibody was then aliquoted and stored at -70°C

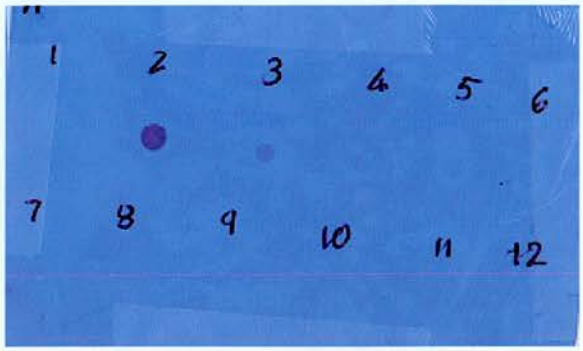


Figure 2.1. Coomassie stained dot-blots of eluate fractions.

Lowry protein assay

This assay is based on that of Lowry *et al.*, 1951, and was carried out on a 96-well ELISA plate (Life Technologies Ltd). The reagents listed in appendix 1 were made up using distilled water.

The unknown protein sample(s) was/were diluted in triplicate in 5ml tubes with 0.1M NaOH to give a final volume of 200µl each (a 1:40 dilution worked well for protein G column eluates). 1ml of alkaline carbonate solution was added to all samples and BSA protein standards (appendix 1) and the tubes were vortexed to mix. Tubes were left to stand for 10 minutes. 100µl of 1M Folin and Ciocalteu's phenol reagent was added to all tubes. The tubes were vortexed and left to stand for 30 minutes. 200µl of each BSA protein standard and sample was added to an ELISA plate and read at 570nm on a MR 5000 microplate reader (Dynatech Laboratories, Billinghurst, West Sussex, UK). Plate loading order was as detailed in the Lowry assay software file "kimlowry.asy" (BioLinx 2.20, Dynatech Laboratories). The software assay file drew a standard curve and calculated the protein concentration of the unknown samples.

Phenotyping of cells

Flow cytometry was used to phenotype cultured cells and assess the purity of cell cultures used in experiments. The presence or absence of the cell surface antigens listed in table 2.1 was used to identify cells as monocytes, macrophages, dendritic /Langerhans cells, T-cells or B-cells.

Cells for phenotyping were taken from culture (a 30 minute incubation with sterile 0.02% (w/v) EDTA and gentle cell scraping, if required, was used on adherent cell populations). Cells were placed in a round bottomed 96 well plate in aliquots of about 10^5 cells per well. The plate was centrifuged at 100*g* for 3 minutes, and the supernatant discarded. The cells were washed by re-suspension in 200µl of chilled flow buffer and a second centrifugation. The supernatant was again removed and the cells were re-suspended in 10µl of the primary antibody solution. The primary antibodies and the concentrations used are listed in table 2.1. The cells were incubated with the primary antibody for 30 minutes on ice. Cells were then washed as above and resuspended in 10µl of the secondary antibody (R-PE-conjugated goat $F(ab')_2$ anti-mouse Ig (Dako, Cambridge, Cambridgeshire, UK) at 67µg ml^{-1} in flow buffer) if the primary antibody was not fluorochrome conjugated. Cells were incubated with secondary antibody for 30 minute on ice in the dark, and then washed in flow buffer. After staining cells were re-suspended in 200µl of fresh flow buffer and transferred to labelled flow cytometry tubes containing 200µl of flow fix. Cells were either put through the flow cytometer immediately or kept at 4°C in the dark for up to 2 weeks.

Antigen	Clone	Supplier	Conc. used / μg ml^{-1}	Isotype
CD1a	NA1/34	Dako	50	IgG2aκ
CD3	UCHT-1	Immunotech	1:10	IgG1κ
CD3-FITC	UCHT-1	Dako	Neat (100)	IgG1κ
CD4	MT310	Dako	10	IgG1κ
CD4-FITC	QS4120	Sigma	Neat	IgG1
CD8$^{\alpha}$-FITC	30324X	Pharmingen	Neat	IgG1κ
CD14	UCHM1	P. Beverley	28	IgG2a
CD18	MHM23	Dako	10	IgG1κ
CD40	11E9	Novocastra	1:40	IgG1
CD45 (ICAM-1)	T29/33	Dako	13	IgM
CD54	15.2	Novocastra	1:40	IgG1
CD80 (B7.1)	BB-1	B. D.	50	IgM
HLA DR,DP,DQ (β chain)	CR3/43	Dako	8	IgG1κ
Isotype control	UPC 10	Sigma	-	IgG2aκ
Isotype control	MOPC	Sigma	-	IgG1κ

Table 2.1. Details of primary antibodies used for phenotype cells. Monoclonal antibodies were supplied by Dako, Sigma, Becton Dickinson / Pharmingen, Coulter-Immunotech (Luton, Bedfordshire, UK), and Novocastra Laboratories (Newcastle, Tyne and Wear, UK) or prepared from supernatants of hybridoma cell lines from Prof. Sir Peter Beverley (The Edward Jenner Institute for Vaccine Research, Compton, Berkshire, UK). Concentrations used are stated as μg ml^{-1} or, were absolute concentration was not stated by the manufacturer, as dilutions from supplied stock. Three of the antigens listed above were FITC conjugated and not used with a secondary reagent.

Quantification of cell-surface antigen levels

For the antigens CD4 and CD18 flow cytometry was used to not only detect the presence or absence of the antigen but to quantify the relative level of antigen on different macrophage populations. So that increases as well as decreases in antigen level could be seen reproducibly, an appropriate primary antibody concentration for use in subsequent experiments was determined by carrying out a titration of antibody concentration. Staining was as described above and results from the antibody titration tests are presented in chapter 4.

Annexin V staining

Apoptotic cells loose their membrane-bilayer asymmetry; negatively charged phospholipids normally on the internal face of the membrane appear on the external face. Annexin V can bind these lipids; if this annexin V is FITC conjugated apoptotic cells can be selectively stained with this reagent. The intact membrane-impermeant dye propidium iodide (PI) can be used in conjunction with annexin V staining to indicate necrotic cells. This technique was carried out using the Pharmingen Annexin V-FITC apoptosis detection kit (Becton Dickinson UK Ltd.).

A suspension of at least 1×10^5 cells was washed twice in cold PBS and then resuspended in 100µl of binding buffer. 5µl of annexin V-FITC and 2µl of PI (as supplied) was added to the cells, which were mixed and incubated in the dark at room temperature. 400µl of binding buffer was then added to the cells, which were analysed by flow cytometry within 1 hr. Unstained and singly stained cells from each specimen were also analysed.

Microscopy methods

Standard light microscopy

Cells in culture were regularly examined and counted on a haemocytometer (Sigma) by a CK2 phase contrast microscopy (Olympus, London, UK).

FITC conjugation of protein

Gp120 was conjugated to the fluorochrome FITC using the following protocol based on that from Holmes *et al.*, 1997. The gp120 was supplied sterile and its sterility was retained by using only sterile reagents (listed in appendix 1) for the conjugation. Solutions were filter sterilised if required. Note that the success of conjugation is highly dependent on the quality of the reagents used. The FITC and DMSO used must be anhydrous. Because these substances are hydroscopic, small quantities were purchased fresh before beginning the experiment.

A fresh vial of R5-tropic gp120 or X4-tropic $gp120_{IIIB}$ (NIBSC Centralised Facility for AIDS Reagents) containing 50µg of gp120 was made up to 500µl with distilled

water. The gp120 was then loaded into a Slide-A-Lyser™ dialysis cassette (Pierce & Warriner, Chester, Cheshire, UK) as per manufacturer's instructions. The gp120 was dialysed for 2 days at 4°C against 4 changes of 500ml of FITC labelling buffer. The gp120 was removed from the Slide-A-Lyser™ to a 5ml tube and 2µl of 5µg ml^{-1} FITC in DMSO was added to the gp120. The gp120 / FITC was left for 2 hours in the dark at room temperature to allow conjugation to take place. The labelled gp120 was loaded into a new Slide-A-Lyser™ and dialysed against 4 changes of 500ml of final dialysis buffer at 4°C for 2 days. The product was removed and the FITC:gp120 ratio determined. FITC conjugated gp120 was stored at -20°C until required.

Determination of FITC:gp120 ratio. The molar ratio of FITC to gp120 should be between 6 and 20. This is so it is comparable to the molar ratio of FITC to protein of the control protein, FITC-BSA, which had a ratio of 11.2. If the ratio was too low then the fluorescent signal would have been too weak for detection. If there was too much FITC bound to the gp120, the likelihood of the gp120 losing its binding properties because of steric hindrance would gave been increased. The FITC:gp120 ratio was determined by taking spectrophotometer measurements of a sample of FITC conjugated gp120.

Using a PU 8620 spectrophotometer (Philips, Cambridge, UK) and a Quartz (UV transparent) cuvette a sample of FITC-gp120 was diluted in final dialysis buffer, and absorption at 492nm was measured after blanking the instrument with final dialysis buffer.

The following formulae (Holmes *et al.*, 1997) were used to calculate the molar ratio of FITC:gp120 in FITC-gp120.

$$\text{moles gp120} = \frac{\text{mg ml}^{-1}\text{ protein}}{1.2 \times 10^5} \qquad \text{Moles FITC} = \frac{\text{Abs}_{492}}{6.9 \times 10^5}$$

$$\text{molar ratio} = \frac{\text{moles FITC}}{\text{moles gp120}}$$

Staining of macrophages for fluorescence microscopy

Monocyte-derived human macrophages were grown from Buffy coats as detailed above, except that 1ml of cells were plated onto 70% ethanol-sterilised glass

coverslips (BDH, Poole, Dorset, UK) sitting in the bottom of 6 well plates; by this method macrophages differentiated whilst adhering to the glass cover-slips. All media formulations, cell-washing stages, cell densities and phenotypes were as previously described. After 6 days of differentiation, cells were incubated with FITC-conjugated gp120 (see above) or FITC conjugated BSA (Sigma) for various lengths of time. Cells were then fixed by removal of their medium and the addition of 1ml of 2% paraformaldehyde in PBS to each well for 20 minutes at room temperature. At this stage fixed coverslips could be stored for several months at -20°C in coverslip racks; if this was done, cells were defrosted by 5 minutes of immersion in 2% paraformaldehyde in PBS at room temperature.

After fixation, cells were washed three times in PBS in 6 well plates. Cells were then permeabilised by the addition of 0.1% Triton X-100 in PBS at 1ml per well for 5 minutes at room temperature. Cells were washed thrice in PBS. Cells were blocked to prevent non-specific binding of antibody with 1ml per well of 0.2% BSA in PBS at room temperature for 10 minutes. Cells were stained with directly fluorochrome-conjugated and/or primary and secondary antibodies as detailed in table 2.2 (see below). Antibodies were diluted in flow buffer (see above) and all fluorochrome conjugates were kept in the dark. 100µl of each antibody was added for 30 minutes at room temperature and followed by three PBS washes. Antibody was added using the inverted coverslip method described in figure 2.2. Coverslips were carefully floated off the Parafilm™ after staining and washed (cell side up) in PBS in 6 well plates. After staining, a final 3 washes were carried out in 6 well plates with distilled water. Coverslips were then dabbed dry at the edges and mounted on glass slides (BDH) using Vector-shield anti-photo-bleaching glycerol-based mounting medium (Vector Labs Ltd., Orton Southgate, Cambridgeshire, UK). The edges of the coverslips were sealed with nail varnish (Boots the Chemist, Nottingham, UK) and the slides stored in the dark at 4°C for up to 2 weeks before examination.

Antigen	Clone	Supplier	Conc. used / μg ml^{-1}	Conjugate
HLA DR	DA6 231	K. Guy	3	Non-conjugated
CD4	MT310	Dako	1.8	FITC
Mouse IgG	Goat polyclonal	Sigma	1:100 from stock	TRITC

Table 2.2. Details of antibodies used to stain macrophages for fluorescence microscopy. See table 2.1 caption and method above for details of suppliers. Anti-CD4 was directly conjugated to a fluorochrome and cells were stained in a single stage at the concentration used. HLA-DR staining was a two-stage process, utilising the goat anti-mouse reagent as a secondary antibody. HLA-DR was always stained before CD4.

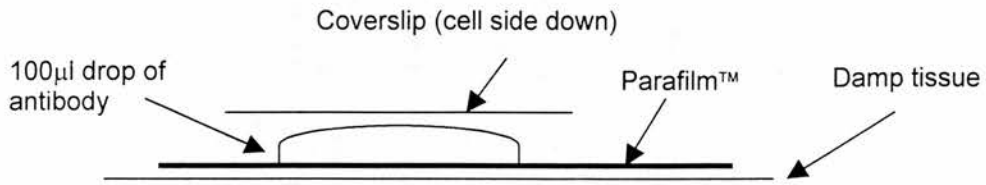


Figure 2.2. The inverted coverslip method of staining. A 100μl drop of the diluted antibody was added to a piece of Parafilm™, which was placed on a damp paper tissue in a plastic tray, the coverslip to be stained was then carefully lowered, taking care to avoid trapping air bubbles, onto the drop with the cell side facing down. The coverslip was then incubated at room temperature for 30 minutes. In order to avoid creating a vacuum under the coverslip, which would distort the cells, the coverslip was gently floated off the Parafilm™ with PBS.

Fluorescence microscopy

Stained cells were visualised on an Axiophot fluorescent microscope (Carl Ziess Ltd, Welwyn Garden City, Hertfordshire, UK) using FITC and TRITC filter sets. This microscope was used to check specimens before examining them by confocal microscopy.

Laser-scanning confocal microscopy

Stained macrophages were visualised on a DMRE laser scanning confocal microscope with a TCS NT image capture computer system (Leica Microsystems,

Heildelberg, Germany). Images were saved to CD-ROM as tagged image files (.tif) and analysed by the TCS NT system and Scion Image (Scion Corp., Frederick, MD, USA). Invaluable technical assistance in the capture of confocal images was provided by Linda Sharp, Department of Biomedical Sciences, University of Edinburgh.

Immunoassays

Cytokine measurements

IL-10, IL-12 and TGF-β1 levels were determined in cell culture supernatants using Quantikine® enzyme-linked immuno-sorbant assay (ELISA) kits (R&D Systems Europe Ltd) as described in the manufacturer's instructions. Plates were washed using a HandyWash manual microplate washer (Dynatech Laboratories) and read at 450nm on a Revelation 3.04 microplate reader (Dynex Technologies, Billinghurst, West Sussex, UK) with the correction wavelength set to 550nm.

All samples and standards were set up in duplicate as recommended by the manufacturer. The plate reader software automatically drew standard concentration curves and calculated cytokine concentrations of unknown samples.

For IL-10 and IL-12 determination supernatants were diluted 1:13 in calibrator diluent before being assayed. For TGF-β1 determination it was necessary to first activate the samples to liberate immunoreactive TGF-β1 from its latent complexes. This was achieved by 10 minutes of room temperature incubation with 0.17M HCl, followed by neutralisation with 0.17M NaOH and 71mM HEPES.

Prostaglandin measurements

PGE_2 in cell culture supernatants was immunoassayed by Vivien Grant (MRC Centre for Reproductive Biology, Edinburgh).

Nucleic acid purification methods

DNA isolation

Genomic DNA was isolated from PBS washed frozen pellets of 1-5×10^6 PBMCs using the Wizard® Genomic DNA purification Kit (Promega, Southampton, Hampshire, UK). Cell structure was disrupted by 4 freeze-thaw cycles using liquid nitrogen and a 95°C water bath. Nuclear Lysis Solution from the Wizard® kit was then added to the cell sample and the manufacturer's protocol followed from this point onwards.

RNA isolation

Cellular RNA was isolated from samples using the RNeasy spin column kit as per the manufacturer's instructions (Qiagen Ltd, Crawley, West Sussex, UK). The first cell lysis buffer (RTL) was added to a pellet of cells or, in the case of adherent cells, directly to the cells in the tissue culture vessel after removal of the medium.

DNase treatment of RNA. The majority of RNA was not DNase treated because the RNA isolation method used gave very low levels of DNA contamination and for PCR experiments, where DNA contamination could have given misleading results, intron-spanning, message-specific primers or appropriate controls were used. However, when RNA was isolated from HIV-1 infected cultures, the RNA was DNase treated as a safety precaution to eliminate possibly infectious HIV-1 pro-virus. Only after this had been completed, was the RNA allowed out of the category-three pathogen-containment facility. To the 50µl volume of RNA, 10µl of 5×RT buffer from the Expand RT® kit (Roche Diagnostics Lewes, East Sussex, UK) was added and the solution mixed. 10U of DNase I (FPLC*pure*®, Amersham Pharmacia Biotech) was then mixed with each RNA sample. The sample was incubated at room temperature with the DNase for 30 minutes. The DNase was then inactivated by the addition of 5µl of 25mM EDTA (Life Technologies Ltd) and heating in a waterbath to 65°C for 10 minutes.

Nucleic acid quantification

Extracted RNA and DNA were quantified using a GeneQuant II photospectrometer (Amersham Pharmacia Biotech) and UV-transparent quartz cuvettes. The sample dilution was varied in order to give 260nm absorbency reading of at least 0.010 and preferably of at least 0.020. RNase free water was used as diluent and to 'zero' the instrument. Quantified RNA was stored at -70°C, DNA at -20°C.

Polymerase chain reaction (PCR) methods

Bioinformatics and primer design

The sequences used for PCR primers came from publications or from Dr Marilyn Moore and Donald Innes (PPL Therapeutics, Roslin, Midlothian, UK) as detailed in Appendix 2. All sequences used were verified against the GenBank database held at the National Center for Biotechnology Information web site (http://www.ncbi.nlm.nih.gov/). Where possible, message specific, intron-spanning primers were used. Primers were synthesised by Oswel DNA Service (Southampton, Hampshire, UK) or Life Technologies Ltd.

Reverse transcription (RT)

cDNA was synthesised using the Expand RT® kit (Roche Diagnostics). All other reagents were purchased from Promega UK. Ideally, 1μg of RNA was reverse transcribed; however, in many cases the amount of RNA available was below this and 0.2μg or 0.1μg of RNA was used instead. For semi-quantitative applications, an equal mass of each RNA was used in the experiment. For each sample the following reagents were added to a labelled thin-walled 0.5ml Eppendorf® tube (Advanced Biotechnologies Ltd, Epsom, Surrey, UK):

RNA	1μg, 0.2μg or 0.1μg
15mer oligo dT (final concentration 0.05g l^{-1})	1.5μl
RNase free water	variable volume
Total volume	8.5μl

Tubes were vortexed to mix, and micro-centrifuged (momentary pulse to 10^4 rpm) to gather contents at the bottom of the tube. Contents were denatured by heating for 10 minutes to 65°C on an Omn-E™ or OmniGene™ thermal cycler (Hybaid, Teddington, Middlesex, UK). Oligo dT was then annealed to the poly A tail of mRNA by cooling the tubes on ice for at least 2 minutes. The following reaction mix was made up and 11.5µl of it was added to each tube, which was then vortexed and centrifuged as above:
(Note, quantities given below were multiplied by the number of reaction tubes.)

5× Expand RT® buffer	4µl
100mM dithiothreitol	2µl
3.3mM of each dNTP	4µl
RNasin® ribonuclease inhibitor	0.5µl (20 Units)
50 Unit μl^{-1} Expand RT® enzyme (added last)	1µl
Volume per sample	11.5µl

The tubes were then heated to 42°C for 1 hour on a thermal cycler to allow the RT reaction to proceed. Synthesised cDNA was stored at -20°C for up to several years.

Basic PCR protocol

PCR was carried out using *Taq* Supreme DNA polymerase kits (Helena BioSciences, Sunderland, Tyne and Wear, UK) and an Omn-E™ or OmniGene™ thermal cycler with tube temperature control (Hybaid). All other reagents were purchased from Promega UK. 2µl of each cDNA or 2µl (approximately 0.1µg) of each genomic DNA of interest (the 'template') was aliquoted into a separate, labelled, thin-walled 0.5ml Eppendorf® tube (Advanced Biotechnologies Ltd) for each primer pair used. The following 'master mixes' were made up for each amplicon of interest:
(Note, quantities given below were multiplied by the number of reaction tubes which was the number of templates under investigation plus an addition tube with no template which was used as a negative control.)

RNase free water	14.5μl
10× $MgCl_2$-free *Taq* buffer	2μl
3.3mM of each dNTP	0.2μl
25μM sense primer	0.4μl
25μM anti-sense primer	0.4μl
100mM MgCl (final concentration of 1.5mM)	0.3μl
5Unit μl^{-1} *Taq* DNA polymerase (added last)	0.2μl
Volume per tube	18μl

18μl aliquots of 'master mix' were added to each template tube; the 18μl of each master mix left over was retained as a negative control to be run alongside the other samples. All tubes were vortexed and centrifuged to gather contents at the bottom of the tube and a single drop of molecular grade mineral oil (Sigma) was added to each tube. All tubes were placed on a thermal cycler and the appropriate cycling program (see below) was run.

PCR programs

The following programs were run on an Omn-E™ or OmniGene™ thermal cycler with tube temperature control (Hybaid)

hiv and *β-actin*	(94°C	45s
	55°C	35s
	68°C	2min 30s) × *n* cycles (variable)
ccr5Δ32 and *β-actin*	(94°C	1min
	55°C	1min
	72°C	1min 30s) × 5 cycles
	(94°C	30s
	60°C	30s
	72°C	45s) × 35 or *n* cycles
il-10 and *β-actin*	94°C	5min
	58°C	5min
	(94°C	1min
	58°C	2min
	72°C	3min) × *n* cycles (variable)

cd4 and *β-actin*	(94°C	1min
	58°C	2min
	72°C	2min) × *n* cycles (variable)
il-12p40 and *β-actin*	(94°C	45s
	55°C	45s
	72°C	45s) × *n* cycles (variable)
	72°C	10min

DNA gels

PCR gels were made up and run in Tris / borate / EDTA (TBE) buffer, see appendix 1 for recipes.

Agarose (Sigma) gels from 1 to 3.5% (w/v) were made by mixing agarose with an appropriate volume of 1×TBE buffer and heating the mixture in a microwave oven until the agarose had dissolved. The gel was then allowed to cool until it was still molten but cool enough to hold with the bare hand. Ethidium bromide was then mixed with the molten gel to give a final concentration of 100mg l^{-1}. The gel was cast into a mould using an appropriate sample comb to form loading wells. When set, the comb was removed and the gel transferred to an electrophoresis tank containing the recommended volume of TBE as running buffer. 2μl of loading dye (0.25% (w/v) orange G (molecular grade, Promega), 50% (v/v) glycerol, 50% (v/v) RNase / DNase free water) was added to 10μl of each DNA sample, which was then loaded into a well on the gel. pGEM® molecular weight markers (Promega) were run in one lane to allow for PCR product size determination. Markers were added as recommended by the manufacturer.

Electrophoresis was at no more than 70 mA, until the dye-front had migrated a sufficient distance towards the anode for DNA fragment size to be resolved.

Gel documentation

Gels were visualised by UV trans-illumination and images were captured, saved to floppy disk and printed using the Enhanced Analysis System (EASY, version 4.19, Scotlab, Coatbridge, Lanarkshire, UK)

Semi-quantitative PCR

'Semi-quantification' of relative mRNA level was carried out as devised by Wang *et al.*, 1989. This method attempts to equate relative band intensity on a PCR gel with relative level of a specific mRNA in the starting material. An equal mass of each RNA was reverse transcribed. A variable cycle number PCR reaction was carried out to determine the optimum number of cycles required for a near-linear relationship between the RNA level and resultant DNA band intensity. All cDNAs were then amplified by PCR for this number of cycles and the band intensities as determined by the EASY were recorded. To allow for errors in the initial equalisation of RNA mass between samples, samples were compared as ratios of the intensity of a band amplified from the gene of interest to the intensity of the *β-actin* housekeeping gene band from the same cDNA.

Real-time (kinetic) RT-PCR

This technique was carried out in collaboration with Professor Rodney Kelly and Gail Baldie (MRC Centre for Reproductive Biology, Edinburgh) using a ABI Prism 7700 sequence detector (PE Biosystems, Warrington, Cheshire, UK). All reagents and consumables for use with this technique were purchased from PE Biosystems. There are several kinetic PCR systems in use (Higuchi *et al.*, 1992; Higuchi *et al.*, 1993); these studies used the PE Biosystems TaqMan® system (http://www.pebio.com/ab/about/pcr.html, Holland *et al.*, 1991; Fouchier *et al.*, 1992; Livak *et al.*, 1995), which is based around amplifying a specific sequence between two unlabelled primers during a thermal cycling program, as in conventional PCR. To allow a target-specific signal to be detected concomitantly with amplification, an oligonucleotide 'TaqMan®' probe is designed to anneal to the target sequence between the sense and anti-sense primers at a T_m higher than the T_m of the primers. The TaqMan® probe is chemically modified to be non-extendable at the 3' end and labelled with a fluorescent reporter dye (FAM or VIC) at the 5' end. The fluorescence of the reporter dye is quenched with a quencher dye (TAMRA) attached seven nucleotides from the 5' end so that the reporter dye is only able to fluoresce when released into the reaction mixture having been separated from the

quencher dye. During PCR amplification cycles *Taq* polymerase extends the primers in a 5' to 3' fashion, and exhibits 5' to 3' exonuclease activity on the TaqMan® probe, this un-quenches the reporter dye and allows the instrument to detect an increase in reporter-dye fluoresce which is proportional to the rate of target amplification. The PCR reactions were carried out in duplicate in 96-well PCR plates fitted with optically transparent caps on a heated lid thermal cycling block. Two amplification reactions were run in each sample-well simultaneously and distinguished by the use of different reporter dyes, The fluorochrome FAM was used to report the amplification of the gene under analysis and the fluorochrome VIC was used to report the amplification of cDNA derived from the 'house-keeping' 18S ribosomal (r) RNA. The intra-cellular level of 18S rRNA is assumed to be constant in a population of non- or asynchronously-dividing cells and was measured as an internal control to compare to the level of mRNA for the gene under analysis. The two reactions were designed so that the 18S amplification was complete by 16 thermal cycles and the amplification of the analyte was beginning in ernest only after 18 thermal cycles, this minimised interference between the two amplification reactions due to competition for dNTPs. Each well was irradiated and its fluorescence monitored by the instrument at the end of each thermal cycle. A third fluorochrome, ROX, was present in the reaction mixture are a passive control to allow automatic compensation for instrument irregularity and reaction volume errors.

Reverse transcription for real-time PCR

RNA was extracted using RNeasy kits as described above. cDNA was synthesised using the MultiScribe RT® kit (PE Biosystems). Ideally, 0.2μg of RNA was reverse transcribed; however, if the amount of RNA available from some samples was below this, 0.1μg of RNA was used instead throughout the whole experiment. The following reaction mix was made up, vortexed then centrifuged:
(Note, quantities given below were multiplied by the number of reaction tubes.)

10× TaqMan® buffer	1μl
25mM $MgCl_2$	2.2μl
2.5mM of each dNTP	2μl
20 Unit μl^{-1} RNase inhibitor	0.2μl
50μM Random hexamer primers	0.5μl
50 Unit μl^{-1} MultiScribe RT® enzyme (added last)	0.25μl
Total volume	6.15μl

Each RNA was added to a 0.5ml Eppendorf® tube and diluted in nuclease-free water to give a volume of 3.85μl. 6.15μl of the reaction mix was added to each tube, which was vortexed and centrifuged before placing on an Omn-E™ thermal cycler with tube temperature control and a heated lid (Hybaid). Tubes were incubated at 25°C for 1 hour, 48°C for 45 minutes and then 95°C for 5 minutes to allow the RNA to be reverse transcribed and then denature the reverse transcriptase. After reverse transcription cDNAs were diluted 5:1 with nuclease free water before proceeding to the PCR step.

Reaction set-up for real-time PCR

PCR was carried out using PE Biosystems TaqMan® Universal PCR Master Mix which is optimised for TaqMan® reactions and contains AmpliTaq Gold® DNA polymerase, AmpErase® UNG, dNTPs with dUTP, passive reference dye 1 (ROX) and optimised buffer components. The 18S rRNA internal control reagents were purchased as a ready optimised mixture and used as recommended by PE Biosystems. All reactions were run in duplicate. 2.5μl of each cDNA diluted as above was aliquoted into two separate wells of a MicroAmp® optical 96 well plate for each primer pair used. A cDNA sample reverse transcribed from RNA extracted from a large batch of the T-47D breast cancer cell line (Keydar *et al.*, 1979) by Gail Baldie and found to be positive for most of the genes of interest was run in duplicate with each primer / probe set as a positive control as was a no-template negative control. The following 'master mixes' were made up for each amplicon of interest, vortexed and centrifuged:

(Note, quantities given below were multiplied by the number of reaction wells, which was twice the number of templates under investigation plus an addition two wells with no template as a negative control and two wells set up with positive control cDNA.)

TaqMan® Universal Master Mix	12.5µl
2.5µM sense primer	3µl
2.5µM anti-sense primer	3µl
5µM TaqMan probe	1µl
Nuclease free water	2.625µl
TaqMan® 18S rRNA control reagent	0.375µl
Volume per well	22.5µl

22.5µl aliquots of 'master mix' were added to each well used to bring the total well volume to 25µl. MicroAmp® optical caps were carefully pressed onto each well and the plate was placed in the ABI Prism 7700 sequence detector running SDS software (PE Biosystems) and using a heated lid. The same cycling program (see below) was run for all primer / probe combinations.

Real-time PCR program

50°C	2min
95°C	10min
(95°C	15s
60°C	1min) × 40 cycles

Primer and probe sequences for real-time PCR

See appendix 2 for details of the primer and probe sequences used. All primers and probe sequences were designed by Professor Rodney Kelly using PrimerExpress® software (PE Biosystems) and verified by PE Biosystems' technical experts. Because of the constraints on primer design imposed by the TaqMan® system and the limited GenBank availability of genomic sequences, most of the primers used were not intron-spanning and could, in theory, have amplified genomic DNA contaminating the cDNA. In order to rule out this possibility, a portion of each RNA investigated

was added to a highly efficient *β-actin* PCR reaction and found to give no detectable product in the absence of a reverse transcription step.

Statistics from real-time PCR

The real time PCR machine took readings of fluorescence at the end of each PCR cycle at wavelengths corresponding to the analyte, 18S housekeeping-control and passive reference fluorochrome-emissions. Figure 2.3 shows a typical set of data as it was presented by the SDS software (PE Biosystems) at the end of a PCR run. Note that the fluorescence statistic (termed R_n) has been normalised relative to the passive reference fluorochrome. The threshold marker was set as high as possible on the curve but before any of the signals began to plateaux. The software then analysed the data and output a pair of 'ct' values for each sample tube. Ct is the number of cycles that each sample needs to produce a signal crossing the threshold; each tube yielded two ct values, $ct_{\text{gene sample } n}$ for the gene of interest and $ct_{\text{18S sample } n}$ for the 18S housekeeping control. The ct values were exported to a Microsoft Excel97 spreadsheet and the following formulae were used to calculate $2^{-\Delta\Delta ct \text{ sample } n}$, which is a value which represents the relative mRNA levels of samples linearly, so that the value of 6 signifies twice as much mRNA as a value of 3.

$$\Delta ct_{\text{sample } n} = ct_{\text{gene sample } n} - ct_{\text{18S sample } n}$$

$$\Delta\Delta ct_{\text{sample n}} = \Delta ct_{\text{sample n}} - \Delta ct_{\text{untreated control}}$$

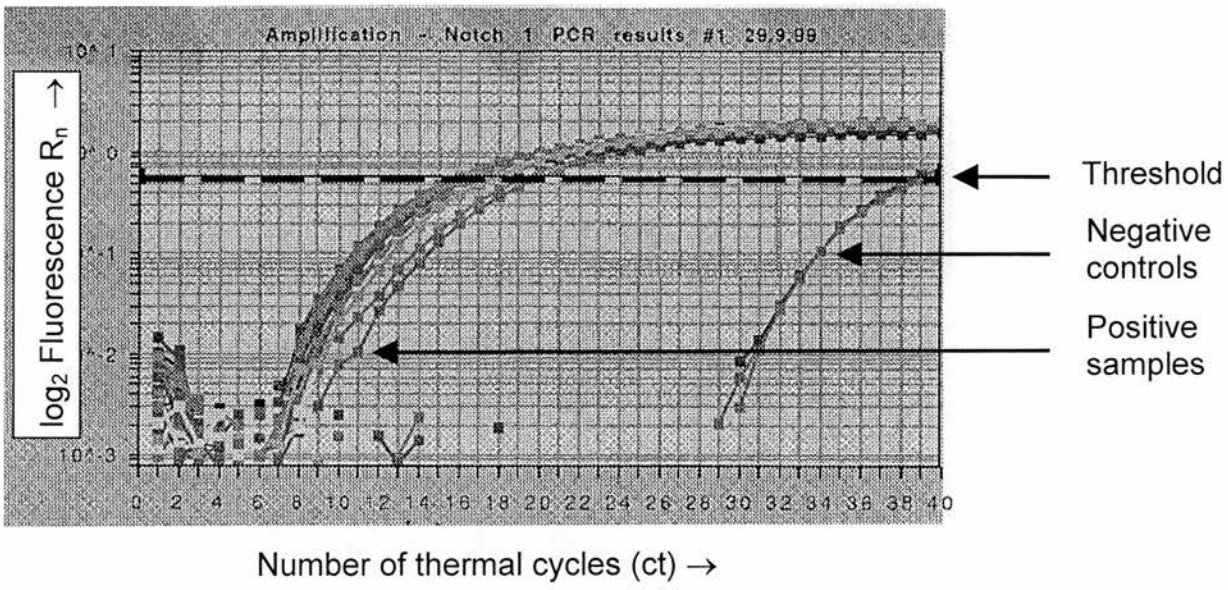


Figure 2.3. Typical real time PCR data. These data shows the 18S housekeeping signal from a set of samples and negative controls. It can be seen that as the samples undergo an increasing number of thermal cycles, the fluorescence of the tubes increases in line with the built up of specific PCR product. It can also be seen that the signals from the negative control wells cross the line at about 39 cycles, this is expected and is due to background signal caused by of non-specific probe cleavage.

CHAPTER 3: RESULTS

DISTRIBUTION OF THE CCR5Δ32 GENOTYPE

Background

The discovery of HIV-1 coreceptors

Very soon after the discovery of HIV-1, the cell surface molecule CD4 was identified as the virus' primary entry receptor (Dalgleish *et al.*, 1984; Klatzmann *et al.*, 1984; Maddon *et al.*, 1986). However, from these early days it was suspected that CD4 alone was not sufficient to allow HIV-1 entry (Clapham *et al.*, 1991) and the hunt for coreceptors which would explain cellular tropism (see introduction) began. After some false starts such as mistaking complement receptor 3 for HIV-1's coreceptor (Stoiber *et al.*, 1997), it was shown that a chemokine receptor is the coreceptor on T-cells and APCs (Dragic *et al.*, 1996; Deng *et al.*, 1996; Choe *et al.*, 1996; Doranz *et al.*, 1996; Rottman *et al.*, 1997; Jones *et al.*, 1998; Albright *et al.*, 1999, reviewed in D'Souza and Harden, 1996; Weiss, 1996; Ross *et al.*, 1999). M-tropic viruses, important in sexual transmission and initial infection, generally use CCR-5 as a coreceptor. T-tropic viruses generally use CXCR-4 (Follis *et al.*, 1998; Tscherning *et al.*, 1998). An alternative classification scheme groups viral strains as being R5-tropic or X4-tropic. M(R5)-tropic viruses can infect both macrophages and T-cells because both cell types express CCR-5. Although both cell types express CXCR-4, T-tropic viruses that use this coreceptor are usually only able to infect T-cells. It appears that macrophage CXCR-4 is unavailable for binding by many T(X4)-tropic viruses possibly due to differences in its formation of multimers (Lapham *et al.*, 1999a; Lapham *et al.*, 1999b), or tyrosine sulphation (Farzan *et al.*, 1999).

Ccr-5 *coreceptor gene polymorphism*

Chemokine receptors are members of the seven-transmembrane-span, serpentine, G-protein-binding family of receptors which includes the β-adrenergic receptor and bacteriorhodopsin. As discussed in the introduction, genetic polymorphisms of chemokine receptor genes have been described (Smith *et al.*, 1997; Quillent *et al.*, 1998; EugenOlsen *et al.*, 1998; Lee *et al.*, 1998; Martin *et al.*, 1998a; Magierowska *et al.*, 1999; Su *et al.*, 1999; Mariani *et al.*, 1999). Eight polymorphisms have been identified in the *ccr5* gene (AnsariLari *et al.*, 1997). The most common polymorphism in Caucasian populations is the *ccr5Δ32* frame-shift deletion. This mutation results in the synthesis of an incomplete molecule of CCR-5 and a failure of cell-surface expression, making the carrier effectively null for surface CCR-5 (Rana *et al.*, 1997, figure 3.1). Homozygosity for the *ccr5Δ32* allele was discovered in a high proportion of people repeatedly exposed to HIV-1 but who had failed to become infected (Samson *et al.*, 1996; Liu *et al.*, 1996; Hoffman *et al.*, 1997). More recently it has been shown that *ccr5Δ32* homozygosity is not the only factor which can account for HIV-1-exposed but uninfected (EU) individuals, as EU people are also present in ethnic groups in which the *ccr5Δ32* allele is absent (Li *et al.*, 1997; Plummer *et al.*, 1999; Kaul *et al.*, 1999).

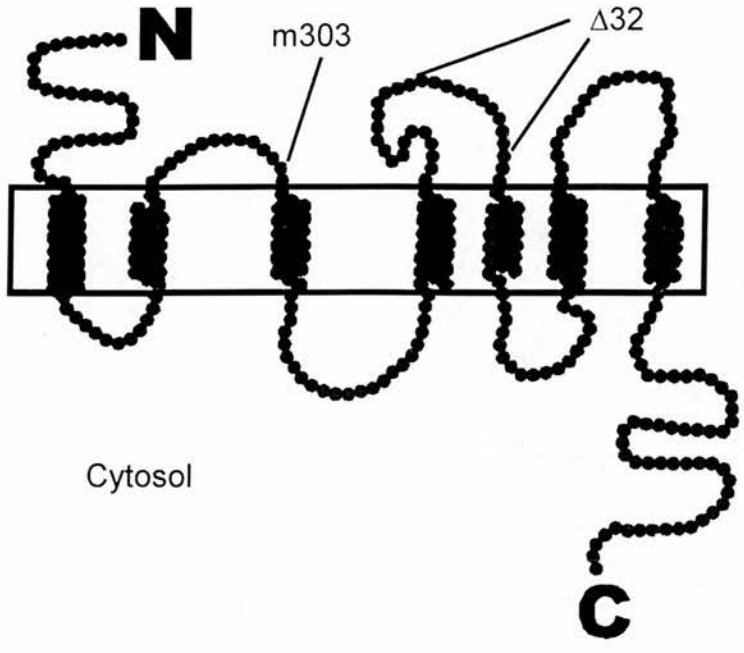


Figure 3.1. Diagram of the structure of CCR-5 showing likely configuration in the plasmalemma. The position of the *m303* premature stop mutation (Quillent *et al.*, 1998) and the *Δ32* deletion mutation are marked. Neither of these mutations allows a complete molecule of CCR-5 to reach the plasmalemma.

Diagram adapted from Quillent *et al.*, 1998. © The Lancet 1998.

Ccr5Δ32 *homozygous cells are protected from infection by M-tropic HIV-1*

In vitro infection assays have been used to show that primary cultures of homozygous *ccr5Δ32* T-cells and monocyte derived macrophages are resistant to infection by the M-tropic CCR-5-ultilising HIV-1 strains BAL, SF162 and JR-FL. T (X4)-tropic strains HXB and IIIB were able to infect T-cells but not macrophages. The dual (R5X4) tropic HIV-1 strain 89.6 was able to infect both *ccr5* null macrophages and T-cells by utilising CXCR-4 (Rana *et al.*, 1997). The observation that macrophage CXCR-4 is accessible to some dual tropic HIV-1 strains and inaccessible to T-tropic strains is in line with work identifying two distinct mechanisms for dual-tropism (Yi *et al.*, 1999). Additional data will be presented

below which confirm the observation that HIV-1_{BAL} is unable to infect monocyte-derived macrophages homozygous for *ccr5Δ32*.

Heterozygous PBMC and T-cells express slightly reduced levels of CCR-5 and, on average require a higher viral dose to be infected *in vitro* with M-tropic HIV-1 (Kim *et al.*, 1998; Paxton *et al.*, 1999).

The ccr5Δ32 *allele protects HIV-1 infected individuals from rapid disease progression*

Homozygosity for the *Δ32* allele at the *ccr5* locus protects individuals from HIV-1 infection. Evidence for this comes from epidemiological studies of HIV-1 transmission in homosexual men (Huang *et al.*, 1996; Paxton *et al.*, 1998), injecting drug abusers (Alvarez *et al.*, 1998), haemophiliac recipients of infected clotting factors (Wilkinson *et al.*, 1998; Kupfer *et al.*, 1999), and babies born to HIV-1 infected mothers (Philpott *et al.*, 1999). While heterozygosity at the *ccr5* locus does not confer protection from infection (Edelstein *et al.*, 1997; Rousseau *et al.*, 1997; Husman and Schuitemaker, 1998; Misrahi *et al.*, 1998; Mangano *et al.*, 1998), it limits the patient's viral load (Katzenstein *et al.*, 1997; Buseyne *et al.*, 1998) and slows progression of HIV-1 disease, at least in the early stages of infection when M-tropic HIV-1 predominates (Huang *et al.*, 1996; Dean *et al.*, 1996; Husman *et al.*, 1997; Rappaport *et al.*, 1997; Paxton *et al.*, 1998; Misrahi *et al.*, 1998; Bratt *et al.*, 1998). Reduced risks for the appearance of several opportunistic infections including toxoplasmosis (Meyer *et al.*, 1999), and non-Hodgkin's lymphoma (Dean *et al.*, 1999) and AIDS dementia complex (VanRij *et al.*, 1999) have also been reported in *ccr5Δ32* heterozygotes.

Although the correlation between *ccr5Δ32* and protection from rapid disease progression has been demonstrated independently many times, it is not the sole determinant of progression rate or transmission risk (Cohen *et al.*, 1997b) and there are a small number of studies (e.g., Schinkel *et al.*, 1999) which fail to show heterozygous protection from progression. Although such people are rare, several HIV-1^+ individuals have been identified who are homozygous for *ccr5Δ32* (Balotta *et al.*, 1997; O'Brien *et al.*, 1997; Theodorou *et al.*, 1997; Biti *et al.*, 1997; Husain *et*

al., 1998; Michael *et al.*, 1998; Kuipers *et al.*, 1999; Heiken *et al.*, 1999). Invariably, when HIV-1 isolated from these individuals is phenotyped, it is found to be CXCR-4 tropic (whether or not it has the unusual ability to use CXCR-4 on macrophages has not been reported). Why CXCR-4 tropic HIV-1 is not usually transmitted is poorly understood. It could be that macrophage / dendritic cell infection that is usually CCR-5 mediated is required for efficient transmission across an anal / genital mucous membrane. This explanation does not account for why *ccr5Δ32* homozygotes are protected from intra-venous transmission of HIV-1.

Other phenotypes of the ccr5Δ32 *allele*

In healthy individuals in the absence of HIV-1 infection, the *ccr5Δ32* allele, in either its homozygous or heterozygous form does not confer any phenotype. The immune system acts normally without CCR-5 even though it functions as a receptor for the pro-inflammatory chemokines MIP-1α, MIP-1β and RANTES (Combadiere *et al.*, 1996). This is presumable due to redundancy in the chemokine system.

Increased levels of MIP-1α have been detected in cerebrospinal fluid of multiple sclerosis (MS) patients; however, CCR-5 deficiency fails to protect against MS (Bennetts *et al.*, 1997), or against insulin dependent diabetes mellitus, another disease associated with pro-inflammatory chemokines (Philpott *et al.*, 1999). However, the *ccr5Δ32* allele does influence some of the clinical variables (including a reduction in the degree of joint stiffness and presence of IgM rheumatoid factor) of rheumatoid arthritis (Garred *et al.*, 1998; GomezReino *et al.*, 1999).

Aims of chapter

- To undertake a meta-analysis of data on the global distribution of the *ccr5Δ32* allele from published sources to demonstrate the reported high incidence of *ccr5Δ32* in north European populations.

- To use statistical methods to analyse this data in such as way as to be able to make a prediction of *ccr5Δ32* incidence in south east Scotland.

- To *ccr5* genotype blood donors attending sessions of the SNBTS in south east Scotland to find the actual allele frequencies in comparison to the prediction.

- To validate the infection assay used in this thesis by confirming that the CCR-5-tropic strain HIV-1_{BAL} is unable to infect homozygous *ccr5Δ32* macrophages.

Methods

See chapter 2 for details of procedures mentioned below.

Literature search

Reported *ccr5Δ32* allele frequencies for various populations were obtained from 28 different published papers and tabulated in a standard form alongside the approximate latitude of each population's position on the globe (Wilett, 1985).

Hardy-Weinberg equilibrium calculations

Allele frequencies were calculated from reported genotype frequencies. Expected genotype frequencies were calculated on the assumption that the alleles were in Hardy-Weinberg equilibrium (HWE) as described in Connor and FergusonSmith, 1993, and Mange and Mange, 1989. The Chi-squared test (Excel97, Microsoft) was then used to compare actual genotype frequencies with the expected frequencies if HWE applied. The results of this test calculated to the 95% confidence level, were then reported as a Yes/No answer to the question, "are the *ccr5Δ32* and *wt* alleles at HWE in this population?"

Regression analysis

Regression analysis was performed in order to correlate allele frequency and latitude data. Because allele frequency is proportional data it cannot be used directly in regression analysis. Data was first transformed using the logistic (logit) transformation. The logit transform of each reported allele frequency (y) was then correlated against the latitude (x) by Bill Adams (University of Edinburgh Medical Statistics Unit) using weighted regression and Genstat 5.4.1 software.

Ccr5Δ32 *genotyping*

Blood donors were genotyped for *ccr5Δ32* using a PCR-based method, which could be applied equally successfully to cDNA or genomic DNA. cDNA was usually used because it had been synthesised for use in other experiments. RNA was isolated from PBMC from Buffy coats from South East Scotland blood donor sessions (SNBTS). This RNA was then reverse-transcribed to cDNA. Alternatively, genomic DNA was isolated from blood donor PBMC samples. Using either cDNA or genomic DNA and primers detailed in chapter 2 and appendix 2, PCR was used to amplify a section of the *ccr5* gene spanning the *Δ32* deletion site. When the PCR products were electrophoresed on a 2.5% agarose DNA gel the *ccr5Δ32* mutant allele could be distinguished from the *wildtype* allele by a size difference. This allowed homozygous *wildtype*, homozygous *Δ32* and heterozygous individuals to be identified and counted.

HIV-1$_{BAL}$ Infectivity assay

HIV-1$_{BAL}$ was incubated with wildtype or homozygous *ccr5Δ32* monocyte-derived macrophages for 96 hours, as described in chapter 2, before a PCR-based assay was used to detect the presence of HIV-1 mRNA (indicative of infection). 32 thermal cycles were used for the PCR reaction, a number determined to be optimal for the detection of infection (figure 6.2).

Results

Ccr5Δ32 *allele frequency*

The *ccr5Δ32* allele is not evenly distributed either geographically or across ethnic groups. Tables 3.1 to 3.6 show the global distribution and HWE status of the *ccr5Δ32* allele compiled from various sources.

The mutant allele has been reported to be present in all European populations at a frequency of 0.9% (Corsica) to 15.5% (Poland, table 3.1). Regression analysis was used to examine the relationship between mutant allele frequency and latitude of population (°N). Figure 3.2 shows the result of this analysis, which indicate significant correlation between the variables. The regression line predicts an allele frequency of 12.61% for South East Scotland (56°N), with a 95% confidence interval of 8.42% to 16.80%.

In the vast Ural-Volga region and Northern Asia (table 3.2) incidence of the *ccr5Δ32* allele is very variable. The mutation is present in both Caucasian members of this region and non-Caucasian groups such as the Tartars. The mutant allele frequency in some of these non-Caucasian groups is so high that it is unlikely to be the result of intermarriage with Caucasian settlers. This data, therefore, dispels the myth that the *ccr5Δ32* allele is exclusively present in Caucasian populations. The mutant allele is however, completely absent in Oriental and East Asian populations.

The mutation is absent from African populations (Table 3.3) with the exception of Mediterranean Africans. The highest reported incident in an African population was in a group of Moroccans, Algerians and Tunisians living in Paris. No Africans were found homozygous for the defective allele.

In the Middle East the mutation was almost completely absent from all groups except Israeli Jews. Caucasian Jews had the highest incidence of *ccr5Δ32* (table 3.4).

The incidence data for the Asia Pacific is shown in table 3.5. In East Asia the mutation is absent. There is a low mutation incidence in many populations from the Indian sub-continent. Aboriginal Australians have an allele frequency of near zero, but Caucasian Australians have a frequency similar to that of Northern or Central Europeans. A similar situation exists in the Americas (table 3.6); native populations

and African and Asian settlers have a *ccr5Δ32* allele frequency of zero or very low (this could be due to intermarriage with Caucasians). Caucasian Americans have an allele frequency similar to Europeans.

Out of 127 populations tested for HWE, 8 (6.3%) showed deviation from this at the 95% confidence level. However at this confidence level one would expect about 6.4 (5% of 127) false negatives. The HWE test may not be an appropriate test for small samples and those that consist of a mixture of groups.

In South East Scotland out of 94 blood donors genotyped (figure 3.3) 70 were homozygous *wildtype*, 20 were heterozygous and 4 were homozygous mutant. This gives a *ccr5Δ32* allele frequency of 14.89% and a *wildtype* allele frequency of 85.11. If the alleles were at HWE, one would expect 69.06 *wt/wt*, 23.87 *Δ32/wt* and 2.06 *Δ32/Δ32* genotypes. The Chi-squared test shows that the deviation from this expected result is not significant (P=0.114), thereby providing evidence that the alleles are in HWE in South East Scotland's blood donors.

Table 3.1 *ccr5Δ32* mutation frequencies in West and Central Europe (Caption appears after table 3.6)

Region	Country	Group (BD = Blood donors)	No. tested	*ccr5Δ32* genotype frequency			*ccr5Δ32* allele % freq.	HWE?	Latitude	Reference
				+/+	+/-	-/-				
	Scotland	South East Scotland BD	94	70	20	4	14.9	Yes	56°N	Reported data
West and Central Europe	France	Ajaccio (Corsica) BD	104	102	2	0	0.9	Yes	42.8°N	Lucotte and Mercier, 1998b
	Italy	Sardinia	241	234	7	0	1.4	Yes	39.5°N	Magierowska *et al.*, 1998
	France	Basque BD	111	107	4	0	1.8	Yes	43°N	Lucotte and Mercier, 1998b
	Greece	Greeks	63	60	3	0	2.4	Yes	38°N	Martinson *et al.*, 1997
	Bulgaria	Gypsies living in Bulgaria	47	Data not given			3.2	Yes	43°N	Stephens *et al.*, 1998
	Italy	Sardinians	100	92	8	0	4.0	Yes	40°N	Libert *et al.*, 1998
	Greece	Athens	143	131	12	0	4.1	Yes	38.5°N	Magierowska *et al.*, 1998
	Cyprus	Cypriots	84	77	7	0	4.2	Yes	35°N	Martinson *et al.*, 1997
	Greece	Greeks	160	Data not given			4.4	Yes	43°N	Stephens *et al.*, 1998
	Bulgaria	Bulgarians	29	Data not given			4.5	Yes	43°N	Stephens *et al.*, 1998
	Ireland	Irish	44	40	4	0	4.6	Yes	53.5°N	Martinson *et al.*, 1997
	Spain	San Sebastian	100	90	10	0	5.0	Yes	43°N	Magierowska *et al.*, 1998
	France	Nice and Grasse BD	124	112	11	1	5.2	Yes	43.8°N	Lucotte and Mercier, 1998b
	Portugal	Portuguese born	124	111	13	0	5.2	Yes	39°N	Lucotte and Mercier, 1998a

(continued over)

Table 3.1 continued

Region	Country	Group (BD = Blood donors)	No. tested	*ccr5Δ32* genotype frequency			*ccr5Δ32* allele % freq.	HWE?	Latitude	Reference
				+/+	+/-	-/-				
West and Central Europe	Bulgaria	Sofia	75	67	8	0	5.3	Yes	43°N	Magierowska *et al.*, 1998
	Italy	Italians	172	Data not given			5.5	Yes	43°N	Stephens *et al.*, 1998
	Italy	Italians	91	81	10	0	5.5	Yes	43°N	Martinson *et al.*, 1997
	Italy	North Italy born	62	55	7	0	5.6	Yes	45°N	Lucotte and Mercier, 1998a
	Italy	Milan BD	122	108	13	1	6.1	Yes	45.5°N	Balotta *et al.*, 1997
	Spain	Basques	89	79	9	1	6.2	Yes	42.8°N	Libert *et al.*, 1998
	Turkey	Turks	104	91	13	0	6.3	Yes	39°N	Libert *et al.*, 1998
	Turkey	Turks	40	Data not given			6.3	Yes	38°N	Stephens *et al.*, 1998
	Portugal	Portuguese	101	88	13	0	6.4	Yes	39°N	Libert *et al.*, 1998
	France	Perpignan Catalan BD	102	90	10	2	6.8	No	42.8°N	Lucotte and Mercier, 1998b
	Spain	Catalans	98	84	14	0	7.1	Yes	41.4°N	Lucotte and Mercier, 1998a
	Slovenia	Slovenians	110	Data not given			7.7	Yes	46°N	Stephens *et al.*, 1998
	Switzerland	Swiss BD	399	340	54	5	8.0	Yes	47°N	Yang *et al.*, 1999
	France	Paris	50	44	4	2	8.0	No	49°N	Magierowska *et al.*, 1998
	Spain	Catalans	49	41	8	0	8.2	Yes	41.2°N	Martinson *et al.*, 1997
	Albania	Albanians	73	Data not given			8.2	Yes	41°N	Stephens *et al.*, 1998

(continued over)

Table 3.1 continued

	Country	Group (BD = Blood donors)	No. tested	*ccr5Δ32* genotype frequency			*ccr5Δ32* allele % freq.	HWE?	Latitude	Reference
				+/+	+/-	-/-				
Region	Denmark	Danes	24	Data not given			8.3	Yes	56°N	Stephens *et al.*, 1998
	Sweden	Saami	120	101	18	1	8.3	Yes	67°N	Libert *et al.*, 1998
	Switzerland	Berne born	64	54	9	1	8.5	Yes	47°N	Lucotte and Mercier, 1998a
	Hungary	Hungarian	99	84	13	2	8.6	Yes	47°N	Libert *et al.*, 1998
	Spain	Basques	29	24	5	0	8.6	Yes	42.8°N	Martinson *et al.*, 1997
	France	Champagne-Ardennes BD	276	234	36	6	8.7	No	48.5°N	Lucotte and Mercier, 1998b
	Italy	Italians in Milan	98	82	15	1	8.7	Yes	45.5°N	Libert *et al.*, 1998
	Austria	Austrians	36	Data not given			8.9	Yes	48°N	Stephens *et al.*, 1998
	France	French	230	Data not given			8.9	Yes	47°N	Stephens *et al.*, 1998
	Finland	Finns	195	Data not given			9.1	Yes	60°N	Stephens *et al.*, 1998
	Belgium	Belgiums	704	582	114	8	9.2	Yes	51°N	Samson *et al.*, 1996
	Germany	Munich University staff and students	421	348	67	6	9.4	Yes	48°N	Malo *et al.*, 1998
	Spain	Murcia Spaniards	100	81	19	0	9.5	Yes	38°N	Libert *et al.*, 1998
		Individuals of 'West European Heritage'	122	96	24	0	9.8	Yes		Liu *et al.*, 1996
	Spain	Spaniards	56	Data not given			9.8	Yes	40°N	Libert *et al.*, 1998

(continued over)

Table 3.1 continued

Region	Country	Group (BD = Blood donors)	No. tested	*ccr5Δ32* genotype frequency			*ccr5Δ32* allele % freq.	HWE?	Latitude	Reference
				+/+	+/-	-/-				
West and Central Europe	France	Bordeaux/Montpellier/Marseilles BD	326	265	58	3	9.8	Yes	44°N	Lucotte, 1997
	France	Montpellier	99	79	20	0	10.1	Yes	43.5°N	Libert *et al.*, 1998
	Czech Rep.	Czechs	161	Data not given			10.2	Yes	52°N	Stephens *et al.*, 1998
	Norway	Norwegian	100	79	21	0	10.5	Yes	60°N	Libert *et al.*, 1998
	Germany	Mülheim born	99	70	17	2	10.6	Yes	51°N	Lucotte and Mercier, 1998a
	Germany	German	208	Data not given			10.8	Yes	51°N	Stephens *et al.*, 1998
	France	Lille BD	101	81	18	2	10.9	Yes	51°N	Lucotte and Mercier, 1998b
	Denmark	Danes	100	78	22	0	11.0	Yes	56°N	Libert *et al.*, 1998
	France	Breton	100	79	20	1	11.0	Yes	48°N	Libert *et al.*, 1998
	Hungary	Gypsies living in Hungary	104	Data not given			10.1	Yes	47°N	Szalai *et al.*, 1998
	England	English	283	223	57	3	11.1	Yes	53°N	Martinson *et al.*, 1997
	Hungary	Budapest	94	73	21	0	11.1	Yes	47°N	Magierowska *et al.*, 1998
	Hungary	Non-HIV pead. patients in Budapest hospital	280	222	54	4	11.1	Yes	47°N	Szalai *et al.*, 1999
	France	Nancy and Strasbourg BD	291	233	51	7	11.2	Yes	48.7°N	Lucotte and Mercier, 1998b
	Ireland	Irish	31	Data not given			11.3	Yes	53.5°N	Stephens *et al.*, 1998
	Hungary	Hungarians	253	Data not given			11.3	Yes	47°N	Szalai *et al.*, 1998

(continued over)

Table 3.1 continued

Region	Country	Group (BD = Blood donors)	No. tested	*ccr5Δ32* genotype frequency			*ccr5Δ32* allele % freq.	HWE?	Latitude	Reference
				+/+	+/-	-/-				
West and Central Europe	Lithuania	Lithuanians	283	220	61	2	11.5	Yes	55°N	Libert *et al.*, 1998
	France	French	422	Data not given			11.7	Yes	54°N	Stephens *et al.*, 1998
	Denmark	Danes	239	185	49	5	12.3	Yes	56°N	Lucotte and Mercier, 1998a
	France	Paris and Paris region BD	294	224	64	6	12.9	Yes	48.9°N	Lucotte and Mercier, 1998b
	Poland	Poles	30	Data not given			13.3	Yes	52°N	Stephens *et al.*, 1998
	Slovakia	Slovakians	30	Data not given			13.3	Yes	49°N	Stephens *et al.*, 1998
	Estonia	Estonians	158	Data not given			13.3	Yes	59°N	Stephens *et al.*, 1998
	France	Brest BD	107	82	21	4	13.5	Yes	48.9°N	Lucotte and Mercier, 1998b
	Denmark	Caucasian, Copenhagen BD (111) & lab staff	151	121	37	2	13.6	Yes	56°N	Garred *et al.*, 1998
	Sweden	Swedes	131	Data not given			13.7	Yes	60°N	Stephens *et al.*, 1998
	Norway	Oslo	83	61	21	1	13.8	Yes	60°N	Magierowska *et al.*, 1998
	Sweden	Swedes	204	152	46	6	14.2	Yes	60°N	Libert *et al.*, 1998
	Sweden	Stockholm	94	70	21	3	14.3	Yes	59°N	Magierowska *et al.*, 1998
	Iceland	Icelanders	102	75	24	3	14.7	Yes	65°N	Martinson *et al.*, 1997
	Poland	Poznan	58	41	16	1	15.5	Yes	54.5	Magierowska *et al.*, 1998

Table 3.2 *ccr5Δ32* mutation frequencies in Northern Asia / Ural-Volga region (Caption appears after table 3.6)

Region	Country	Group (BD = Blood donors)	No. tested	*ccr5Δ32* genotype frequency			*ccr5Δ32* allele % freq.	HWE?	Latitude	Reference
				+/+	+/-	-/-				
North Asia / Ural-Volga region	Georgia	Georgians	50	Data not given			0.0		42°N	Stephens *et al.*, 1998
	Russia	Eastern Natives: Yakut in Yakutsk	23	23	0	0	0.0		62°N	Yudin *et al.*, 1998
	Russia	E. Natives: Nivkh in Yuzhno-Sakhalinsk	20	20	0	0	0.0		47°N	Yudin *et al.*, 1998
	Mongolia	Mongolians	59	59	0	0	0.0		48°N	Martinson *et al.*, 1997
	Mongolia	Mongolians in Ulaanbaatar	30	30	0	0	0.0		48°N	Galeeva *et al.*, 1998
	China	Chinese	40	40	0	0	0.0		40°N	Stephens *et al.*, 1998
	China	Chinese	91	91	0	0	0.0		40°N	Huang *et al.*, 1996
	Russia	Central Asia Natives: Tuvan in Kyzyl	77	76	1	0	0.6	Yes	52°N	Yudin *et al.*, 1998
	Russia	Chukchi and Eskimo in Anadyr	35	34	1	0	1.4	Yes	65°N	Yudin *et al.*, 1998
	Russia	Eastern Natives: Koryak in Palanga	91	87	4	0	2.2	Yes	59°N	Yudin *et al.*, 1998
	Kazakhstan	Kazakh	50	Data not given			3.0	Yes	48°N	Stephens *et al.*, 1998
	Uzbekistan	Uzbek	29	Data not given			3.4	Yes	41°N	Stephens *et al.*, 1998
	Russia	Bashkir	205	190	15	0	3.7	Yes	58°N	Doenhoff, 1999
	Azerbaijan	Azerbijanis	40	Data not given			5.0	Yes	40°N	Stephens *et al.*, 1998
	Russia	Turkic Natives: Chuvash	79	71	8		5.1	Yes	60°N	Galeeva *et al.*, 1998
	Russia	West Siberian Natives Ugric: Mansi	29	26	3	0	5.2	Yes	61°N	Yudin *et al.*, 1998

(continued over)

Table 3.2 continued

Region	Country	Group (BD = Blood donors)	No. tested	*ccr5Δ32* genotype frequency			*ccr5Δ32* allele % freq.	HWE?	Latitude	Reference
				+/+	+/-	-/-				
North Asia / Ural-Volga region	Russia	West Siberian Natives Finnic: Mordvinian	51	45	6	0	5.9	Yes	61°N	Galeeva *et al.*, 1998
	Russia	West Siberian Natives Finnic: Komi	50	44	6	0	6.0	Yes	61°N	Galeeva *et al.*, 1998
	Daghestan	Daghestanis	110	96	14	0	6.4	Yes	42°N	Martinson *et al.*, 1997
	Russia	West Siberian Natives Finnic: Mari	47	38	9	0	9.6	Yes	61°N	Galeeva *et al.*, 1998
	Russia	Udmurt	46	38	7	1	9.8	Yes	58°N	Martinson *et al.*, 1997
	Russia	Russians in Novosibirsk	53	43	9	1	10.4	Yes	55°N	Yudin *et al.*, 1998
	Russia	Udmurt	52	41	9	2	11.5	Yes	58°N	Galeeva *et al.*, 1998
	Russia	Turkic Natives: Tartars	50	Data not given			12.0	Yes	60°N	Stephens *et al.*, 1998
	Russia	West Siberian Natives Ugric: Khanty	61	49	9	3	12.3	No	61°N	Yudin *et al.*, 1998
	Russia	Turkic Natives: Tartars	93	68	25	0	13.4	Yes	60°N	Galeeva *et al.*, 1998
	Russia	Russians	50	Data not given			13.6	Yes	60°N	Stephens *et al.*, 1998
	Russia	Russians	83	61	21	1	13.9	Yes	60°N	Libert *et al.*, 1998
	Russia	Mordvinians	86	58	28	0	16.3	Yes	62°N	Libert *et al.*, 1998
	Russia	St Petersburg	22	22	11	0	16.6	Yes	60°N	Magierowska *et al.*, 1998
	Russia	W. Sib. Nat. Finnic: Komi/Mordvinian/Mari	14	11	1	2	17.9	No	61°N	Yudin *et al.*, 1998

Table 3.3. *ccr5Δ32* mutation frequencies in Africa (Caption appears after table 3.6)

Region	Country	Group (BD = Blood donors)	No. tested	*ccr5Δ32* genotype frequency			*ccr5Δ32* allele % freq.	HWE?	Latitude	Reference
				+/+	+/-	-/-				
Africa		West African Blacks	40	40	0	0	0.0			Zimmerman *et al.*, 1997
	C. Afric. Rep.	Individuals of 'C. African Republic ancestry'	52	52	0	0	0.0		6°N	Martinson *et al.*, 1997
	Gambia	Gambians	56	56	0	0	0.0		14°N	Martinson *et al.*, 1997
	Ivory Coast	individuals of 'Ivory Coast ancestry'	87	87	0	0	0.0		8°N	Martinson *et al.*, 1997
	Kenya	Kenyans	80	80	0	0	0.0		0°	Martinson *et al.*, 1997
	Kenya	Black Kenyans	18	18	0	0	0.0		0°	Huang *et al.*, 1996
	Malawi	Malawians	95	95	0	0	0.0		13°S	Martinson *et al.*, 1997
	Malawi	Black Malawians	13	13	0	0	0.0		13°S	Huang *et al.*, 1996
	Rwanda	Black Rwandans	16	16	0	0	0.0		2°S	Huang *et al.*, 1996
	Tanzania	Black Tanzanians	7	7	0	0	0.0		6°S	Huang *et al.*, 1996
	Uganda	Black Ugandans	45	45	0	0	0.0		1°N	Huang *et al.*, 1996
	Zambia	Zambians	36	36	0	0	0.0		14°S	Martinson *et al.*, 1997
		Sub-Saharan Africans from earlier study	302	301	1	0	0.2	Yes		Lucotte, 1997
	Nigeria	Nigerians	111	110	1	0	0.5	Yes	8°N	Martinson *et al.*, 1997
		Moroccan/Algeria/Tunisian students in Paris	71	62	9	0	6.3	Yes	30°N	Lucotte, 1997

Table 3.4. *ccr5Δ32* mutation frequencies in the Middle East (Caption appears after table 3.6)

Region	Country	Group (BD = Blood donors)	No. tested	*ccr5Δ32* genotype frequency			*ccr5Δ32* allele % freq.	HWE?	Latitude	Reference
				+/+	+/-	-/-				
Middle East	Lebanon	Lebanese	51	Data not given			0.0		33°N	Stephens *et al.*, 1998
	Saudi Arabia	Saudi Arabians	100	100	0	0	0.0		24°N	Stephens *et al.*, 1998
	Yemen	Yemenis	34	34	0	0	0.0		14°N	Martinson *et al.*, 1997
	Israel	Ethiopian Jews	45	45	0	0	0.0			Kantor and Gershoni, 1999
	Israel	Bedouin	51	51	0	0	0.0			Kantor and Gershoni, 1999
	Israel	Palestinian	8	8	0	0	0.0			Kantor and Gershoni, 1999
	Israel	Middle Eastern Jews (Yemen / Iran / Iraq)	130	127	3	0	1.15	Yes		Kantor and Gershoni, 1999
	Saudi Arabia	Saudi Arabians	241	231	10	0	2.1	Yes	24°N	Martinson *et al.*, 1997
	Israel	N. African Jews (Morocco / Libya / Tunisia)	96	92	4	0	2.1	Yes		Kantor and Gershoni, 1999
	Israel	Druze	87	83	4	0	2.3	Yes		Kantor and Gershoni, 1999
	Israel	Ashkenazi Jews	503	Data not shown			9.7	Yes		Stephens *et al.*, 1998
	Israel	Ashkenazi Jews	103	83	19	1	10.2	Yes		Kantor and Gershoni, 1999

Table 3.5. *ccr5Δ32* mutation frequencies in South and Central Asia and Pacific (Caption appears after table 3.6)

Region	Country	Group (BD = Blood donors)	No. tested	*ccr5Δ32* genotype frequency			*ccr5Δ32* allele % freq.	HWE?	Latitude	Reference
				+/+	+/-	-/-				
Asia Pacific	Vietnam	Vietnamese in Paris	48	48	0	0	0.0			Lucotte, 1997
	Japan	Japanese	248	248	0	0	0.0		38°N	Samson *et al.*, 1996
	India	Bengali ancestry	25	25	0	0	0.0		23°N	Martinson *et al.*, 1997
	India	Tamil BD	46	46	0	0	0.0		11°N	Zimmerman *et al.*, 1997
	India	Indians	13	13	0	0	0.0		23°N	Huang *et al.*, 1996
	Hong-Kong	Hong Kong ancestry	50	50	0	0	0.0		22°N	Martinson *et al.*, 1997
	Taiwan	Taiwanese	83	83	0	0	0.0		23°N	Martinson *et al.*, 1997
	Taiwan	Taiwanese	187	187	0	0	0.0		23°N	Li *et al.*, 1997
	Philippines	Filipino ancestry	26	26	0	0	0.0		11°N	Martinson *et al.*, 1997
	Philippines	Negrito ancestry	30	30	0	0	0.0		11°N	Martinson *et al.*, 1997
	Sri Lanka	Sri Lankans	37	37	0	0	0.0		5°N	Martinson *et al.*, 1997
	Burma	Burmese	67	67	0	0	0.0		22°N	Martinson *et al.*, 1997
	Thailand	Thais	83	83	0	0	0.0		16°N	Huang *et al.*, 1996
	Korea	Koreans	50	50	0	0	0.0		36°N	Stephens *et al.*, 1998
	Borneo	Borneo ancestry	151	151	0	0	0.0		0°	Martinson *et al.*, 1997
	Sumatra	Sumatrans	72	72	0	0	0.0		0°	Martinson *et al.*, 1997

(continued over)

Table 3.5 continued

Region	Country	Group (BD = Blood donors)	No. tested	*ccr5Δ32* genotype frequency			*ccr5Δ32* allele % freq.	HWE?	latitude	Reference
				+/+	+/-	-/-				
Asia Pacific	Andaman Is.	Andaman Islanders	24	24	0	0	0.0		12°N	Martinson *et al.*, 1997
	Australia	Aboriginal ancestry	98	96	0	0	0.0		25°S	Martinson *et al.*, 1997
	Fr. Polynesia	French Polynesians	94	94	0	0	0.0		20°S	Martinson *et al.*, 1997
	Guam	Guam ancestry	59	58	0	0	0.0		12°N	Martinson *et al.*, 1997
	New Guinea	New Guineans	96	96	0	0	0.0		8°S	Martinson *et al.*, 1997
	Fiji	Fijians	17	17	0	0	0.0		17°S	Martinson *et al.*, 1997
	China	Chinese	40	40	0	0	0.0		40°N	Stephens *et al.*, 1998
	China	Chinese	91	91	0	0	0.0		40°N	Huang *et al.*, 1996
	Thailand	Thais	101	100	1	0	0.5	Yes	16°N	Martinson *et al.*, 1997
	India	Healthy donors from almost all India's states	100	99	1	0	1.0	Yes	23°N	Husain *et al.*, 1998
	India	Punjabi ancestry	34	33	1	0	1.5	Yes	31°N	Martinson *et al.*, 1997
	Pakistan	Pakistanis	34	32	2	0	2.9	Yes	30°N	Martinson *et al.*, 1997
	India	Gujarati ancestry	32	30	1	1	4.7	No	23°N	Martinson *et al.*, 1997
	Australia	Caucasian Australians of European descent	168	137	30	1	9.5	Yes	25°S	Bennetts *et al.*, 1997
	Australia	Australian	395	Data not given			11.8	Yes	25°S	Stephens *et al.*, 1998

Table 3.6. *ccr5Δ32* mutation frequencies in the Americas (Caption appears after table)

Region	Country	Group (BD = Blood donors)	No. tested	*ccr5Δ32* genotype frequency			*ccr5Δ32* allele % freq.	HWE?	Latitude	Reference
				+/+	+/-	-/-				
Americas	N. America	Cheyenne Indians	100	Data not given			0.0			Stephens *et al.*, 1998
	N. America	Pima Indians	78	Data not given			0.0			Stephens *et al.*, 1998
	N. America	Pueblo Indians	100	Data not given			0.0			Stephens *et al.*, 1998
	Canada	Canadian Inuit	40	40	0	0	0.0		63°N	Yudin *et al.*, 1998
	Mexico	Nuicholes ancestry	52	52	0	0	0.0		22°N	Martinson *et al.*, 1997
	Brazil	Amerindian ancestry	98	98	0	0	0.0		10°S	Martinson *et al.*, 1997
	Brazil	Tikuna tribe members from Umariçu	47	47	0	0	0.0		4°S	Leboute *et al.*, 1999
	Brazil	Tikuna tribe members from Feijoal	46	46	0	0	0.0		4°S	Leboute *et al.*, 1999
	Brazil	Tikuna tribe members from Vendaval	49	49	0	0	0.0		4°S	Leboute *et al.*, 1999
	Brazil	Tikuna tribe members from Belém	49	49	0	0	0.0		4°S	Leboute *et al.*, 1999
	Brazil	Baniwa tribe members	46	46	0	0	0.0		1°N	Leboute *et al.*, 1999
	Brazil	Kashinawa tribe members	29	29	0	0	0.0		5°S	Leboute *et al.*, 1999
	Brazil	Kanamari tribe members	34	34	0	0	0.0		7°S	Leboute *et al.*, 1999
	Jamaica	Jamaicans	119	119	0	0	0.0		18°N	Martinson *et al.*, 1997
	Haiti	Black Haitians	31	31	0	0	0.0		18°N	Huang *et al.*, 1996
	N. America	Asian BD	164	163	1	0	0.3	Yes		Zimmerman *et al.*, 1997

(continued over)

Table 3.6 continued

Region	Country	Group (BD = Blood donors)	No. tested	*ccr5Δ32* genotype frequency			*ccr5Δ32* allele % freq.	HWE?	Latitude	Reference
				+/+	+/-	-/-				
Americas	N. America	Nuu-Chah-Nulth tribe	38	37	1	0	1.3	Yes		Martinson *et al.*, 1997
	Mexico	Mexicans	42	Data not given			2.4	Yes	26°N	Stephens *et al.*, 1998
	Argentina	Buenos Aires BD	64	61	3	0	2.4	Yes	35°S	Mangano *et al.*, 1998
	N. America	Black BD	294	277	17	0	2.9	Yes		Zimmerman *et al.*, 1997
	Brazil	Random population from diverse urban Brazil	100	93	7	0	3.5	Yes	10°S	Passos and Picanco, 1998
	Mexico	Hispanic Mexican medical students	103	95	7	1	4.4	Yes	26°N	SalasAlanis *et al.*, 1999
	USA	New York BD (95% Caucasian)	637	543	85	9	8.1	No	41°N	Huang *et al.*, 1996
	N. America	Native American BD	87	73	11	3	9.7	No		Zimmerman *et al.*, 1997
	N. America	Caucasian BD	387	300	84	3	11.6	Yes		Zimmerman *et al.*, 1997

Tables 3.1 to 3.6. Global incidence of the *ccr5Δ32* allele. Data was compiled from the multiple published sources cited. Note that classification of samples varied from source to source, some groupings are geographic (such as anonymous blood donors attending a certain donation centre), other authors have made an attempt to classify their samples into ethnic groups (based variously on skin-colour, nationality, surname, recent ancestry or self-identification). Each sample is reported as being in Hardy-Weinberg equilibrium (HWE), at the 95% level of probability, or not. Populations are ranked within each table in order of increasing allele frequency.

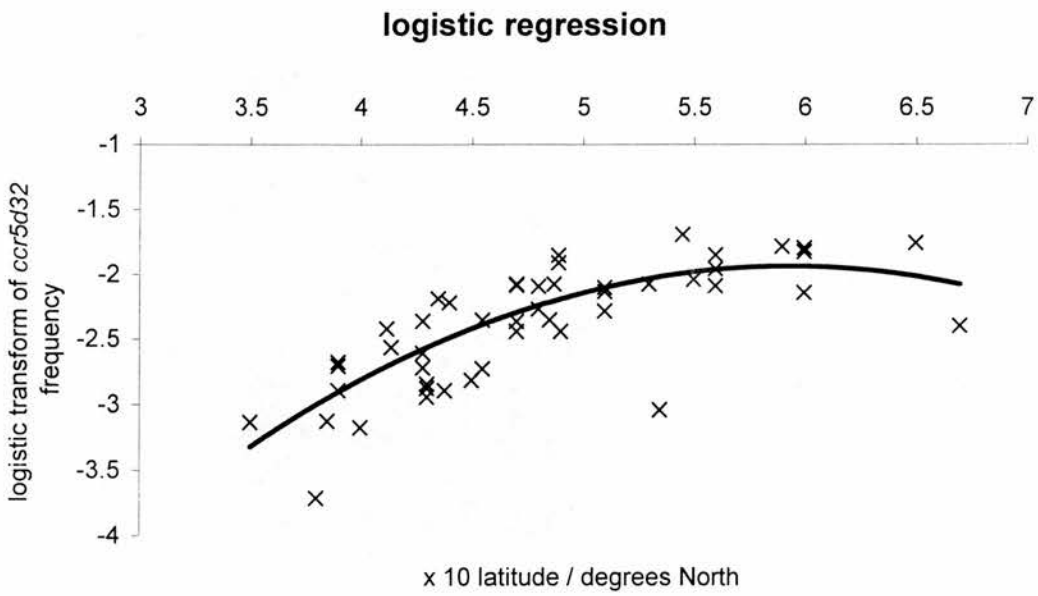


Figure 3.2. Correlation between latitude and *ccr5Δ32* allele frequency. Using data of European incidence of *ccr5Δ32* from table 3.1, and weighted regression, the logistic transform of *ccr5Δ32* frequency was correlated with latitude.

Line of best fit equation $y = -11.24 + 3.139x - 0.2647x^2$

Covariance table				
	constant	1.957929		
	x	-0.759996	0.297069	
	x^2	0.072649	-0.028594	0.002772
		constant	x	x^2

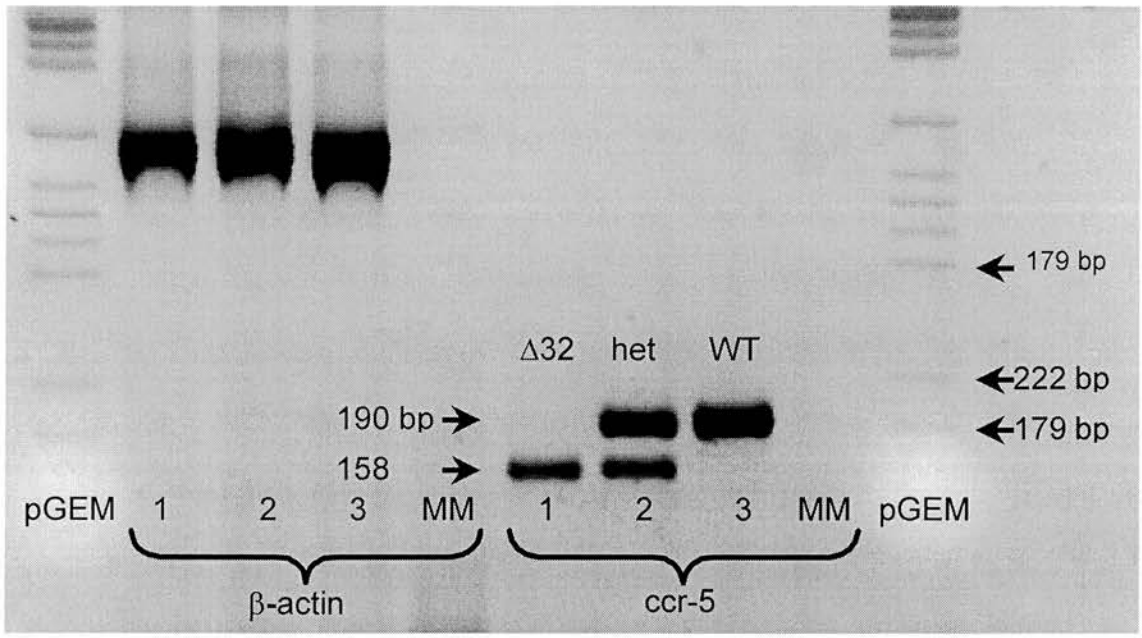


Figure 3.3. Genotyping of *ccr5*. RNA extracted from donors 1, 2 and 3 was subjected to RT-PCR for *β-actin* and *ccr-5*. The *ccr-5* products show a length polymorphism with *wildtype* message amplifying to a 190 base pair product and the *Δ32* mutant message amplifying to a 158 base pair product. This allows sample 1 to be detected as homozygous for the *Δ32* mutation. Sample 2 is heterozygous and sample 3 is homozygous *wildtype*. pGEM = molecular weight marker (Promega), MM = master-mix no-template negative control.

Infection of homozygous ccr5Δ32 *mutant macrophages*

The macrophage (R5)-tropic HIV-1 strain BAL was unable to infect macrophages which were homozygous for *ccr5Δ32* within the 96 hour time-frame allowed by the assay used. Figure 3.4 shows the results of one of these infectivity assays. Macrophages from three homozygous *wildtype* donors and one heterozygous donor were demonstrated to be able to support infection by HIV-1_{BAL}. Macrophages from two donors homozygous for the *ccr5Δ32* mutation were unable to support detectable infection.

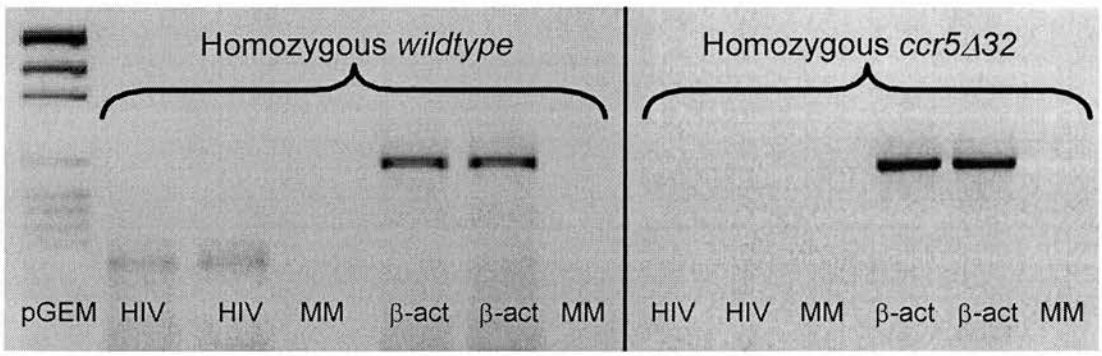


Figure 3.4. HIV-1_{BAL} infectivity assay. An RT-PCR based assay was used to detect mRNA for HIV-1 and *β-actin* from macrophages of the genotype indicated. The figure shows positive signals for both HIV-1 and *β-actin* from RNA extracted from the wildtype macrophages. The mutant macrophages yielded only a signal for β-actin. MM = master mix, no-template negative control for the preceding two lanes. pGEM = molecular weight marker (Promega).

Discussion

CCR-5-tropic HIV-1 can not infect ccr5 *null macrophages*

It has been previously reported that T-cells (Wu *et al.*, 1997; Paxton *et al.*, 1999) and whole PBMC (Quillent *et al.*, 1998) from individuals homozygous for the *ccr5Δ32* mutation are resistant to infection with CCR-5 utilising, M-tropic isolates of HIV-1. This is supported by the observation (figure 3.4) that HIV-1_{BAL}, an M-tropic isolate of HIV-1 is unable to infect homozygous mutant monocyte-derived macrophages. Taken together these data strengthen the evidence for CCR-5 as the major entry coreceptor for HIV-1_{BAL}, The fact that M-tropic HIV-1 isolates require CCR-5 to infect cells and that usually this role of CCR-5 can not be replaced by another coreceptor gives hope for therapeutics aimed at blocking CCR-5 such as those discussed in chapter 6. Because *ccr5* null cells have no known phenotype in healthy individuals, it is hoped that CCR-5 could be functionally blocked with few side effects.

The ccr5Δ32 *mutation is generally in Hardy-Weinberg equilibrium in non-HIV-1 infected populations*

If two alleles A and a have respective population frequencies of p and q and are in Hardy-Weinberg equilibrium (HWE) the frequency of the genotype AA is predicted to be (within random statistical variation) p^2. The frequency of the genotype Aa is $2pq$, and the frequency of aa is q^2 (Mange and Mange, 1989; Connor and FergusonSmith, 1993). HWE is disrupted if there is selective pressure for or against one of the genotypes. Small populations, migration of individuals, non-random mating and populations which are really mixtures of two or more populations may also deviate from HWE. Because the *ccr5Δ32* homozygous genotype is protective against HIV-1, cohorts of HIV-1 infected individuals are not in HWE for this gene. The number of *ccr5Δ32* homozygotes is less than that predicted by the Hardy-Weinberg formula (Michael *et al.*, 1997). Populations that are HIV-1 exposed but uninfected will contain more *ccr5Δ32* homozygotes than predicted and will not be in HWE either (Hoffman *et al.*, 1997; Husman *et al.*, 1997).

Out of the 127 populations meta-analysed (tables 3.1 to 3.6) which contain the *ccr5Δ32* allele, all but eight are in HWE. Because the HWE was calculated to a 95% confidence interval, one would expect about 5% (6.35 out of 127) of the populations to be designated as not in HWE just due to random variation of gene distribution. Also, some of the populations are rather small and some population may be mixtures of several ethnic groupings. Overall, the data from the populations in tables 3.1 to 3.6 would suggest that in populations which have not been significantly exposed to HIV-1, the *ccr5Δ32* allele is in equilibrium with its *wildtype* counterpart and is not currently under any selection pressure. This statement raises a very important question; if there is currently no pressure, apart from HIV-1, for the selection of particular *ccr5* alleles, what forces led to the non-random geographic distribution of the *ccr5Δ32* allele that we see today?

The incidence of the ccr5Δ32 *mutation in SE Scotland is as predicted*

The 14.89% frequency of the *ccr5Δ32* mutation in SE Scotland blood donors is as one would predict for a north European country. It fits within the figure 3.2

regression curve, and is not significantly different from the 11.1% value previously calculated for England (Martinson *et al.*, 1997).

Possible explanations for the distribution of ccr5Δ32

In order to speculate about the origin of the *ccr5Δ32* mutation and the evolutionary forces that lead to its spread and maintenance, it would be useful to know when it arose. The mutation's geographical distribution can give some clues to this. The fact that the mutant allele is present in Caucasian New World populations at similar frequencies to those in the founding European populations (Tables 3.5 and 3.6) would suggest that the mutation arose before mass emigration to the New World from Europe. Genetic studies (Stephens *et al.*, 1998; Libert *et al.*, 1998) using microsatellite repeat sequence data and the coalescence of haplotype theory suggest that the mutation arose, only once in North Eastern Europe about 700 years ago. Nothing in the geographic distribution data presented here contradicts this theory. For a mutation as recent as 700 years old to have spread around Europe with such speed to give the present day distribution patterns, there must have been a strong evolutionary advantage in carrying the mutation. HIV-1 would be a candidate to cause such selective pressure if it had been around hundreds of years ago. HWE data (tables 3.1 to 3.6) show that at the present time, apart from HIV-1, there is no other selective pressure for or against the *ccr5Δ32* mutation. Therefore, it seems likely that an earlier epidemic of a disease for which *ccr5Δ32* provided protection and which is no longer prevalent would have provided the selection force required to explain the mutation's spread. One could argue that such a rapid spread of the mutant allele required a very strong selection force and that an epidemic which was able to exert this force (by extremely widespread and high mortality) would not have escaped historical documentation. Several authors have suggested that the Black Death (The Plague, *Yersinia pestis*) which spread through Europe from 1333 onwards (Ziegler, 1969), arrived in Britain in 1348 (Nikiforuk, 1992) and struck Edinburgh badly in 1568 (Skeyne, 1969) and London in 1665 (the 'Great Plague', Defoe, 1960) could have provided the selection pressure needed for the spread *of ccr5Δ32.* The Black Death certainly produced sufficient levels of mortality with 15 to 50% of Europeans

dying (Nikiforuk, 1992). However, no-one has been able to show that the *ccr5Δ32* allele offers any protection against *Yersinia pestis* or suggest a convincing mechanism of chemokine involvement in the pathogenesis of plague. The disease still strikes today, mainly in the third world (although a small outbreak occurred in Glasgow in 1900, Chalmers, 1901) so there ought to be an opportunity to study the incidence of *ccr5Δ32* genotypes in plague victims and survivors.

Although the plague theory provides for a sufficient die-off at an appropriate date, it does not explain why the *ccr5Δ32* allele did not spread to India, a region ravaged by plague during a similar period to Europe (Ziegler, 1969); maybe there was not time for the mutation to spread this far.

Rhesus macaques (*Macaca mulatta*), sooty mangabeys (*Cercocbus torquatus atys)* and red-capped mangabeys (*Cercocbus torquatus torquatus*) naturally carry SIV. Homozygosity for a 24 base deletion in the *ccr-5* gene (*ccr5Δ24*) of these healthy monkeys results in no functional CCR-5 being produced and offers protection from CCR-5-tropic SIV strains (Chen *et al.*, 1998; Palacios *et al.*, 1998). It seems more likely that the *ccr5Δ24* monkey allele was maintained as a defence against SIV than *Yersinia pestis* as there is no evidence that these monkeys ever occurred in plague regions (Szalay and Delson, 1979). Although the monkey *ccr5Δ24* mutation arose independently of the human *ccr5Δ32* mutation, it provides evidence that primate retroviruses have long been using CCR-5 for entry and that the hosts have responded to this ancient threat by removing the receptor from the path of the virus. This theory is compatible with the view that the human *ccr5Δ32* mutation spread as a defence to an ancient human retrovirus.

In conclusion, much more work needs to be done in order to explain with any degree of certainty the reason for the human *ccr5Δ32* mutation. The Plague theory may be the best that has been offered so far but it remains unconvincing in several ways. It seems likely that an ancient epidemic led to the spread of the *ccr5Δ32* mutation but the disease in question remains elusive. Maybe a now dormant retrovirus was responsible or maybe HIV-1 itself was responsible and it is not such a modern virus as we suppose

CHAPTER 4: RESULTS

GP120 INDUCED CD4 LOSS

Background

HIV-1 infection and gp120 causes immune system dysregulation

As reviewed in the introduction to this thesis, HIV-1 causes AIDS by disrupting the anti-pathogen immune response (Rodriguez and Hard, 1995). HIV-1 is able to infect $CD4^+$ T-cells and $CD4^+$ antigen presenting cells (APCs), including macrophages and dendritic cells (BarréSinoussi, 1988; Hewson *et al.*, 1999; Fidler and Rees, 1999). There is also evidence that both T-cell and APC function can be compromised in uninfected cells of HIV-1^+ patients (Wagner *et al.*, 1992; Desbarats *et al.*, 1996; Chirmule and Pahwa, 1996; Karsten *et al.*, 1996; Ng *et al.*, 1996; Heinkelein *et al.*, 1997; Kaneko *et al.*, 1997; Hewson and Howie, 1998). HIV-1 associated alteration of APC function has downstream consequences for the generation of adaptive immune responses. In addition, altered macrophage function has implications for the innate immune response to pathogens. In particular, lack of IL-12 production by HIV-1 infected individuals is associated with increased opportunistic infection (Chehimi and Trinchieri, 1994; Marshall *et al.*, 1999). Although all the proteins of HIV-1 have been implicated in disrupting infected immune cell function (Willey *et al.*, 1992; Rhee and Marsh, 1994; De *et al.*, 1998; Peter, 1998; Hewson *et al.*, 1999) secreted gp120 is arguably the most damaging viral product because it has the potential to dysregulate the function of uninfected $CD4^+$ cells in HIV-1 infected individuals. Gp120 is found in the plasma of HIV-1^+ patients with AIDS or AIDS related complex (ARC), mainly in the form of immune complexes (Oh *et al.*, 1992).

The structure of gp120 determines viral tropism such that gp120 derived from most primary / macrophage-tropic isolates binds to both CD4 and CCR-5 (R5-tropic gp120) while T lymphocyte-tropic variants bind to CD4 and CXCR-4 (X4-tropic

gp120, Wu *et al.*, 1996; Rizzuto *et al.*, 1998; Choe, 1998; Hoffman and Doms, 1999).

Physiological roles of CD4, CCR-5 and CXCR-4

CD4 on the surface of T lymphocytes acts as a ligand for MHC class II on the surface of APCs. Interactions between MHC class II and multiple CD4 molecules stabilise the MHC class II / TCR interaction required for signal one transmission to the T-cell (Sakihama *et al.*, 1995; Janeway and Travers, 1996). CD4 binding of MHC class II causes signals to be transmitted through $p56^{lck}$, a second messenger associated with the cytoplasmic tail of CD4 in T cells (Veillette *et al.*, 1988). CD4 association with $P56^{lck}$ in T-cells prevents the constitutive CD4 internalisation and recycling of CD4 seen in non-lymphoid cells (PelchenMatthews *et al.*, 1991; PelchenMatthews *et al.*, 1992). CD4 on the surface of APCs is not associated with $p56^{lck}$ (Bowers *et al.*, 1997) but on both T-cells and APCs, CD4 acts as a receptor for the chemotactic cytokine IL-16 (Center *et al.*, 1996). IL-16 and gp120 have both been demonstrated to inhibit the mixed lymphocyte reaction by binding to CD4 and disrupting CD4 / TCR / CD3 complexes (Theodore *et al.*, 1996). It has been reported that gp120 can signal through CD4 and chemokine receptors to mimic the chemotactic actions of IL-16 and chemokines. M-tropic gp120 can mediate chemotaxis of $CD4^+$ T-cells by signalling through CD4 (Iyengar *et al.*, 1999). T-tropic gp120 is able to signal through CXCR-4 in a CD4 independent fashion to cause phosphorylation of Pyk2 (Misse *et al.*, 1999) and chemoattraction of $CD4^+$ and $CD8^+$ T-cells (Iyengar *et al.*, 1999; Misse *et al.*, 1999).

HIV-1 induced changes to cell surface phenotype

There have been several reports of gp120 modulating cell surface CD4 levels. Incubating X4-tropic gp120 with $CD4^+$ T-cells leads to loss of surface CD4 after 6 hours, CD4 loss reaches a nadir at 24 hours and starts to recover after 96 hrs (Theodore *et al.*, 1994). CD4 loss reduces the ability of T-cells to exhibit chemotaxis in response to anti-CD3 or anti-CD4 antibodies. The ability to migrate in response to either of these stimuli returns when CD4 levels recover, a process which can be

blocked by cycloheximide, and is therefore likely to involve *de novo* synthesis of CD4 (Theodore *et al.*, 1994). Naturally expressed X4-tropic gp120 from HIV-1_{IIIB} has been shown to cause surface CD4 loss from monocytes over a period of a few days (Wahl *et al.*, 1989). This down-regulation of CD4 resulted in a reduced chemotactic response to IL-16 and was suggested to be due to gp120-induced monocyte to macrophage differentiation (Wahl *et al.*, 1989). Various other authors have demonstrated gp120 induced surface CD4 loss from macrophage or monocyte cell surfaces. Experiments with recombinant gp120 from HIV-1_{IIIB} showed 30% loss of CD4 from *in vitro* derived macrophages after 6-12 hrs of incubation with gp120 (Karsten *et al.*, 1996). This CD4 loss was dependent on TNF-α secretion in response to CD4 cross-linking and the activation of the ras pathway (Karsten *et al.*, 1996; Tamma *et al.*, 1997). Another study showed that X4-tropic gp120 was able to induce a CD4 loss and a deficit in T-cell stimulation in a tuberculin antigen presentation assay (DurrbaumLandmann *et al.*, 1994). Other agents, such as 1,25-dihydroxy vitamin-D3 (Rigby *et al.*, 1990) have been shown to cause CD4 loss from macrophage cell surfaces. LPS induces a CD4 loss via the induction of TNF and IL-1β (Herbein *et al.*, 1995).

There have been no reports of the effect of R5-tropic gp120 on macrophage or monocyte cell surface molecules, nor any reports of X4-tropic gp120 inducing more than 30% loss of surface CD4 as measured by the intensity of flow cytometry staining.

Endocytosis and phagocytosis

Endocytosis is the process of a cell internalising components of its environment, whether they be liquid (pinocytosis – 'cell drinking') or solid. Endocytosis is a very important function of macrophages both immunologically (Janeway and Travers, 1996), and in the clearance of effete, necrotic or apoptotic cells during tissue development and remodelling (Loegering, 1985; Brewton and Maccabe, 1988; Stoll *et al.*, 1989; Stern *et al.*, 1992; Savill *et al.*, 1996; Mitchell *et al.*, 1999; Bird *et al.*, 1999; Moffatt *et al.*, 1999). Endocytosis by an APC allows previously external potential antigens to enter MHC loading machinery and be presented to T-cells in an

immuno-stimulatory or tolerogenic context (Kurts *et al.*, 1998; Allison *et al.*, 1998; Peterson *et al.*, 1999). Endocytosed protein is cleaved into suitable length peptides, which are mainly loaded into MHC class II molecules for presentation to CD4 T-cells. MHC class I molecules are mainly filled with peptides derived from endogenous protein. There is however some leakage of the MHC loading pathways so that a limited amount of exogenously derived peptide can be presented by MHC class I molecules, and endogenously derived peptide can be presented by class II molecules (Bachmann *et al.*, 1995).

Phagocytosis is a from of endocytosis where an exogenous solid is engulfed by a cell. Phagocytosis takes in a greater volume of material in a single 'gulp' than pinocytosis (Cohn and Steinman, 1982; Pratten and Lloyd, 1986) and is mediated by cell surface receptors for the solid being engulfed. Phagocytosis is triggered by receptor recognition of a ligand, whereas pinocytosis is constitutive (Cohn and Steinman, 1982). A wide range of receptors can be involved in receptor-mediated phagocytosis including Fc receptors (Mellman, 1982; Kim and Schreiber, 1999; May *et al.*, 2000) and complement receptors (Kusner and Hall, 1996; Kim and Schreiber, 1999; Webster *et al.*, 2000). The intracellular destinations of endocytosed material can be various; the route of entry and nature of the material influences this (Dermine and Desjardins, 1999; Clemens *et al.*, 2000). If endocytosed material is to be antigenic if must be correctly processed into suitable peptides and then loaded into MHC molecules before being presented at the cell surface (Janeway and Travers, 1996).

Endocytosed material can be detected as vesicles inside the engulfing cell by confocal microscopy (Ojcius *et al.*, 1996). Pinocytic vesicles can be distinguished from phagosomes in that they are usually much smaller and more numerous (Cohn and Steinman, 1982; Pratten and Lloyd, 1986; Hewlett *et al.*, 1994). If endocytosed material is subsequently discovered in an MHC-containing compartment it is possible that presentation of this material will result if it can be suitably processed and loaded into MHC molecules.

Implications of endocytosis by APCs

Because endocytosed material contributes almost entirely to the peptides presented by APCs in MHC class II and also to a far lesser degree to MHC class I held peptides, the efficiency of endocytosis has the potential to influence the efficiency of antigen presentation, particularly to $CD4^+$ T-cells.

Autoreactive T-cells are not all deleted in the thymus nor completely silenced by peripheral tolerance mechanisms (Genain *et al.*, 1994; Fang *et al.*, 1997; Williams *et al.*, 1998). In order for a T-cell to become activated, an above-threshold number of its cell surface TCRs need to be simultaneously engaged (Valitutti *et al.*, 1995). Functional tolerance to many self antigens may result because antigenic epitopes are not normally presented on APCs at densities high enough to stimulate autoimmune T-cell activation. Any process that increases the presentation of autoimmune epitopes has the potential to break this tolerance. Once tolerance of this nature has been broken it may be difficult to re-establish since memory T-cells with lower stimulation thresholds will have been generated (Hurst *et al.*, 1999; Saparov *et al.*, 1999; Bachmann *et al.*, 1999; London *et al.*, 2000). Presentation of endocytosed material might also break functional tolerance because the endocytic pathway may process self-proteins differently to the endogenous antigen pathway, thereby revealing cryptic epitopes.

There have been several reports of autoimmunity in HIV-1 infected patients. T-cell autoimmunity to the CD4 molecule was found in one quarter of an HIV-1 infected Italian cohort, cases of autoimmunity being more common in cohort members with markers for disease progression (Caporossi *et al.*, 1998). Autoantibodies to platelets (Magnac *et al.*, 1990), CD4 (Chams *et al.*, 1988), T-cell receptors (Marchalonis *et al.*, 1997) and Fas (Stricker *et al.*, 1998) have been found in HIV-1 infected individuals; the last three classes of autoantibodies all have the potential to cause a T-cell immune deficit. A change to the efficiency of antigen processing and presentation also has the ability to dysregulate immune responses in less predictable ways than the induction of autoimmunity. Loss of specific immune responses (such as those against *Candida albicans* and tetanus toxoid, Tassinari *et al.*, 1995) and skewing of responses (for example away from VH3 utilising antibodies, Juompan *et al.*, 1998) have been recognised in HIV-1 disease.

Mechanisms such as immune exhaustion of responses to over-presented antigens, presentation of antigens in a tolerogenic environment (e.g., in absence of co-stimulation) and presentation concomitantly to an autoantigen for which a suppressor cell population exists (leading to bystander suppression, see chapter 5) could be evoked to explain such phenomena.

Summary

The experiments described in this chapter compare interactions between R5- and X4-tropic gp120, with $CD4^+$ monocyte derived macrophages from non-infected healthy individuals. Evidence is presented for a novel mechanism of R5-tropic gp120-induced macrophage surface-CD4 loss and internalisation. This loss is rapid, substantial and does not occur with X4-tropic gp120. Confocal microscopy has been used to follow CD4 loss from the surface, and the kinetics and regulation of surface CD4 recovery has been investigated by semi-quantitative RT-PCR.

Specific questions

- Is the ability of gp120 to cause a loss of CD4 from APC surface influenced by the cellular tropism of the virus from which the gp120 was derived?

- Is gp120-induced CD4 loss due to gp120 mimicking IL-16 or MIP-1α, physiological ligands of CD4 and CCR-5?

- Is gp120 induced CD4 loss dependent on CCR-5 expression? Is it observed in *ccr5Δ32* mutant macrophages, which fail to express CCR-5?

- What is the mechanism of gp120 induced CD4 loss?

- Where do the lost CD4 and associated gp120 end up? If it is endocytosed by the APC, what are the likely consequences of this for the APC and immune system as a whole?

- Do CD4 levels recover? By what mechanism?

Materials and methods

See chapter 2 for details.

Reagents and antibodies

Cells were treated with various agents at 8.3nM (equivalent molarity to 1μg/ml of gp120).

$Gp120_{IIIB}$ and **R5-gp120** were obtained from the NIBSC Centralised Facility for AIDS Reagents. These baculovirus expressed recombinant proteins were derived from the T-cell line adapted virus strain HIV-1_{IIIB} (Ratner *et al.*, 1985, GenBank accession number X01762) and from cDNA isolated from a primary macrophage of paediatric AIDS patient MN (Gurgo *et al.*, 1988, GenBank accession number U72495). The gene sequences used to generate both rgp120s were kindly analysed according to published criteria (DeJong *et al.*, 1992; Fouchier *et al.*, 1992) by Dr Peter Simmonds (Laboratory for Clinical and Molecular Virology, University of Edinburgh) and confirmed to be X4-tropic and R5-tropic respectively.

Cytokines. Recombinant IL-16 was obtained from R&D Systems Europe Ltd. MIP-1α was obtained from the NIBSC Centralised Facility for AIDS Reagents.

Anti-CD4. Macrophages were, on occasion, treated with a cross-linking anti-CD4 monoclonal antibody (clone QS4120 from the NIBSC Centralised Facility for AIDS Reagents). This antibody binds to the gp120 binding site of CD4 and does not compete for binding with the MT-310 clone anti-CD4 used to measure CD4 levels (McKeating *et al.*, 1993; Shotton *et al.*, 1995).

Neutralisation of TNF-α. Neutralising monoclonal anti-TNF-α antibody (clone 1825.121) was obtained from R&D Systems Europe Ltd and added to some macrophage cultures at 1μg ml^{-1}, a concentration shown by the manufacturer to give 100% neutralisation.

FITC conjugation of protein. Gp120 was conjugated to fluorescein isothiocyanate (FITC) as described in chapter 2. The FITC:gp120 ratio of the conjugate was determined using a spectrophotometer and found to be 7:1, similar to a FITC:BSA ratio of 11.2:1 for FITC conjugated BSA which was used as a control protein

Macrophage Isolation and Culture. Macrophages obtained from Buffy-coats from single, anonymous, healthy blood donations were cultured for 6 days before the start of experiments. All the donors were genotyped for the *ccr5Δ32* mutation.

Detection of CD4 and CD18 by Flow Cytometry. To detect alterations in CD4 level an anti-CD4 antibody, MT310, which binds to a different epitope of CD4 from that which gp120 binds to was used (McKeating *et al.*, 1993; Shotton *et al.*, 1995). Anti-CD18 (as a control) was also used on occasion. Cultured macrophages were stained to determine surface marker level as described in chapter 2 after various treatments. Relative intensities of cell surface staining were determined by comparing the mean fluorescence intensity of staining, above the background staining of an isotype control, between samples. Suitable concentrations of antibodies were determined by titrations, the results of which are presented in figure 4.1. Means and standard errors were calculated for each treatment and means were compared using unpaired Mann Whitney U tests.

Detection of cell-surface bound gp120. Six day old macrophages were harvested from culture flasks and placed at 10^5 cells per well in a round bottomed 96-well plate, washed twice with flow buffer and pelleted. 10μl of R5-gp120 or $gp120_{IIIB}$ at 1μg ml^{-1} in flow buffer was added to the cell pellet, which was then agitated and incubated on ice for 2 hours. After washing four times with ice-cold flow-buffer, 10μl of 0.5μg ml^{-1} polyclonal sheep anti-gp120 serum was added to the cell pellets (ARP0734, NIBSC Centralised Facility for AIDS Reagents). Cells were incubated on ice for a further 2 hours before a single wash in flow buffer, the addition of 10μl of 10μg ml^{-1} of biotinylated donkey anti-sheep serum (Sigma) and incubation on ice for 1 hour. Following another wash, 10μl of 1μg ml^{-1} R-PE-conjugated strepavidin was added to each well and incubated for 1 hour on ice. Cells were then washed twice in flow buffer and suspended in flow buffer / flow fix, as detailed in chapter 2, for analysis by flow cytometry.

Endocytosis of FITC conjugated antibody. Anti-CD4 (clone QS4120) and FITC-conjugated goat anti-mouse (Fc fragment specific, Sigma) antiserum at 100μg ml^{-1} each were incubated together on ice for two hours. The resultant antibody complex was then added to macrophages at 8.3nM, as was non-pre-incubated FITC-anti-mouse antiserum. Cells were then treated for confocal microscopy as described below.

Staining of macrophages for confocal microscopy. Monocyte-derived human macrophages were grown on sterile glass coverslips for 7 days before treatment with FITC-gp120 or FITC-BSA for various lengths of time. Cells were then fixed, permeabilised, stained for CD4 or HLA DR, and examined by laser scanning confocal microscopy and image analysis software.

Macrophage *ccr-5Δ32* Genotyping by RT-PCR. Carried out as described in chapter 3.

Semi-quantitative RT-PCR. RNA was extracted, and 0.1μg was reverse transcribed from each sample as described in chapter 2. A variable cycle number PCR reaction was carried out to determine the optimum number of cycles required for a near-linear relationship between the RNA level and resultant DNA band intensity (figure 4.2). All cDNAs were then amplified by PCR for this number of cycles, and relative cDNA levels calculated as described in chapter 2. James Logie, a BSc student under my supervision, did much of this work.

Results

Antibody titration

CD4 and CD18 levels on different macrophage populations were quantified by flow cytometry. So that increases and decreases could be reproducibly measured, an appropriate antibody concentration for use in subsequent experiments was determined by carrying out titrations with different concentrations of primary antibody as described in chapter 2. A concentration of 10μg ml^{-1} was chosen for all subsequent experiments with either antibody, based on these titrations. Figure 4.1 shows data from an anti-CD18 titration as an example.

Semi-quantitative RT-PCR optimisation

Figure 4.2 shows data produced from variable cycle RT-PCR experiments. 31 (*CD4* and *β-actin*) and 29 (*ccr5*) were chosen as appropriate numbers of cycles for all future semi-quantitative RT-PCR under similar conditions.

Gp120 induces a loss of macrophage surface CD4

Recombinant gp120 derived from an M (R5)-tropic primary isolate of HIV-1 caused loss of CD4 from the surface of *ccr5Δ32* wildtype macrophages (figure 4.3). The loss of CD4 was apparent from 1 hour following the addition of gp120 and reached a nadir at approximately 3 hours. By 18 hours after gp120 administration, surface CD4 levels began to recover.

CD4 loss is dependent on gp120 tropism and cell surface CCR-5

The extent of gp120-induced surface CD4 loss was dependent on the viral strain from which the gp120 was derived (figure 4.4). $Gp120_{IIIB}$ induced a maximal 15-25% surface CD4 loss at 3 hours, which did not reach statistical significance as judged by the unpaired Mann-Whitney U test at any time-point. R5-tropic gp120-induced surface CD4 loss was, however, more substantial (65-75% at both 1 and 3 hours) and statistically significant at all time-points (versus starting CD4 levels $P=0.0411$ at 1 hour, $P=0.0087$ at 3 hours, $P=0.0431$ at 18 hours). There were also significant differences between the responses to the two gp120s (see figure 4.4 for details). To confirm that substantial CD4 loss was dependent on CCR-5 binding, the same experiment was repeated twice on macrophages from donors homozygous for the *ccr5Δ32* homozygous mutation, which fail to express surface CCR-5 (figure 4.5). On these macrophages neither $gp120_{IIIB}$ nor R5-tropic gp120 was able to induce a significant loss of surface CD4.

M- and T-tropic gp120 can both bind to the macrophage surface

The differences between the R5-tropic gp120 and $gp120_{IIIB}$ in their induction of surface CD4 loss could be due to differences in their ability to bind to macrophage

cell surfaces. In order to explore this question *ccr5Δ32* wildtype macrophages were incubated with either one of the gp120s used, on ice and in the presence of sodium azide in order to inhibit endocytosis. Cells were then washed and surface-bound gp120 was detected using a polyclonal antibody. Figure 4.6 shows that surface bound gp120 was detectable regardless of the HIV-1 strain from which it was derived. It would be wrong to suggest that figure 4.6 shows R5-tropic gp120 binding to macrophage surfaces more strongly than $gp120_{IIIB}$ does. This may well have been the case, but the difference in fluorescence intensity between the two groups of labelled cells was not large, and could be explained by differences in detection efficiencies by the polyclonal anti-gp120 used in this study.

CD4 and CCR-5 ligands IL-16 and MIP-1α fail to induce a CD4 loss

In order to further explore the apparent requirement of CD4 and CCR-5 binding for the surface CD4 loss induced by R5-tropic gp120, wildtype macrophages were incubated with the CD4 ligand IL-16 or the CCR-5 ligand MIP-1α at 8.3nM (the molarity at which R5-tropic gp120 caused a CD4 loss). Figure 4.7 shows that neither $gp120_{IIIB}$, MIP-1α nor IL-16 caused the substantial loss of surface CD4 observed with R5-tropic gp120.

Levels of CD18, a control antigen, remain unchanged

In order to exclude the possibility that gp120-induced CD4 loss was simply a symptom of a more generalised loss of macrophage surface antigens in response to incubation with the possibly cytotoxic R5-tropic gp120, levels of another macrophage surface marker, CD18 were investigated. Neither $gp120_{IIIB}$, R5-tropic gp120 nor cross-linking anti-CD4 were able to cause a change in wildtype macrophage surface CD18 levels, at 1, 3 or 18 hours following addition of the agent under test. Figure 4.8 shows CD18 levels on macrophages following 3 hours of incubation with gp120 or anti-CD4, a time-point when CD4 levels are at their lowest on R5-tropic gp120 treated cells. In all cases treated macrophages displayed similar CD18 levels to untreated macrophages.

Neutralisation of TNF-α does not inhibit CD4 loss

There have been reports of gp120 (Karsten *et al.*, 1996) and LPS (Herbein *et al.*, 1995) induced losses of CD4 being mediated by TNF-α production. In order to investigate this possibility wildtype macrophages were incubated with $gp120_{IIIB}$, R5-tropic gp120 or cross-linking anti-CD4 in the presence of a neutralising dose of anti-TNF-α. Table 4.1 shows that both R5-tropic gp120 and anti-CD4 were able to induce substantial surface CD4 losses, but no changes in CD18 levels. Neutralisation of TNF-α did not abrogate the CD4 losses observed. Table 4.1 also shows CD18 levels measured in the same experiment. None of the cellular treatments caused a change in CD18 level. However, the data suggests that neutralisation of TNF-α may result in a slight reduction in CD18 levels, an observation which was not pursued.

Loss of surface CD4 is accompanied by increased levels of CD4 in an internal MHC class II^+ pool

One explanation for gp120-induced CD4 loss is that gp120 induced the endocytosis of cell-surface CD4. Following incubation with R5-tropic gp120, wildtype macrophages were fixed, permeabilised and stained for surface and intracellular CD4, before being examined using a fluorescence confocal microscope. Figure 4.9 shows that before gp120-treatment most cellular CD4 is at the macrophage surface. Following 3 and 18 hours of incubation with R5-tropic gp120 there is less CD4 visible at the macrophage surface (this is in agreement with the flow cytometry data) and more internal, peri-nuclear CD4. It is not clear from this experiment whether the internal CD4 has been endocytosed from the cell surface, or if it is the product of *de novo* CD4 synthesis. Interestingly at the 1 hour time-point there was no noticeable increase in intracellular CD4 despite flow cytometry reporting a strong decline in surface CD4 levels (figure 4.4). Double CD4 and HLA DR staining of macrophages incubated for 3 hours with R5-tropic gp120 showed that the internal CD4 was colocalised with HLA DR, suggesting presence in the endoplasmic reticulum or Golgi apparatus (figure 4.10).

CCR-5 tropic gp120 is endocytosed by a pathway resembling receptor mediated phagocytosis.

Because of the difficulty in identifying the origin of intracellular CD4, the possibility of gp120 / CD4 / CCR-5 complex endocytosis was investigated by tracking the internalisation of FITC-conjugated gp120. FITC conjugated R5-gp120 and FITC conjugated BSA were incubated with macrophages for various times before fixation, permeabilisation, staining for HLA DR, and confocal imaging. Figure 4.11 shows images from this experiment and table 4.2 summarises the findings. After 20 minutes to 1 hour BSA entered the macrophages as numerous small endocytic vesicles, which resemble pinocytic vesicles. These persisted for at least five hours but by 2 hours there was evidence of BSA-FITC conjugate breakdown in the form of a more diffuse green stain, especially noticeable in the nucleoplasm where HLA DR staining was absent. In contrast, R5-tropic gp120 entered cells mostly as one or two larger and brighter vesicles resembling phagosomes from about 20 minutes; smaller vesicles only became visible from about 1-2 hours. There was evidence of FITC-gp120 conjugate breakdown from 2-3 hours. Some of both endocytosed proteins were detected in HLA DR-containing compartments. FITC-conjugated $gp120_{IIIB}$ entered macrophages in a form resembling the entry of BSA rather than R5-tropic gp120 (figure 4.13). To further investigate the differences between pinocytosis of soluble protein and receptor mediated phagocytosis an additional experiment was undertaken. Macrophages were incubated for 1 hour with FITC-conjugated polyclonal goat anti-mouse IgG immunoglobulin. This protein ought to be unable to bind to the surface of human macrophages; it was endocytosed resulting in the appearance of about 5 to 30 small fluorescently labelled vesicles per cell (figure 4.12a) resembling those formed by the endocytosis of $gp120_{IIIB}$ or BSA (figure 4.11). In contrast, when macrophages were incubated with pre-formed complexes of (FITC-conjugated goat anti-mouse IgG / mouse anti-human CD4 antibodies) which are expected to bind to the surface CD4 of human macrophages, endocytosis resulted in the appearance of large and usually single fluorescent vesicles resembling those formed by internalised R5-tropic gp120 (figure 4.11).

R5-tropic gp120 enters macrophages along with CD4, gp120$_{IIIB}$ and BSA do not

Figure 4.13 shows results from an experiment in which FITC-gp120$_{IIIB}$, FITC-R5-tropic gp120 or FITC-BSA was incubated with wildtype macrophages for 20 minutes, a length of time too short for *de novo* synthesis of CD4 to confuse the results. Cells were then fixed, permeabilised and stained for CD4. The large bright R5-tropic gp120 containing vesicles also contained CD4 (figure 4.13a). The smaller, less bright and more numerous BSA or gp120$_{IIIB}$ containing vesicles did not contain CD4, most of which remained on the macrophage cell surface.

R5-tropic gp120 induces an up-regulation of mRNA for CCR-5 and CD4

Figures 4.14 and 4.15 show that R5-tropic gp120 but not gp120$_{IIIB}$ induced a concomitant and significant up-regulation of CD4 and CCR-5 mRNA transcript. The increased mRNA was detectable from 1 hour after gp120 incubation and persisted for about 18 hours when levels started to decline. Variation between donor responses was greater than in the case of surface CD4 protein decline with significant differences between responses to the different gp120s only observable at the 1 hour time-point.

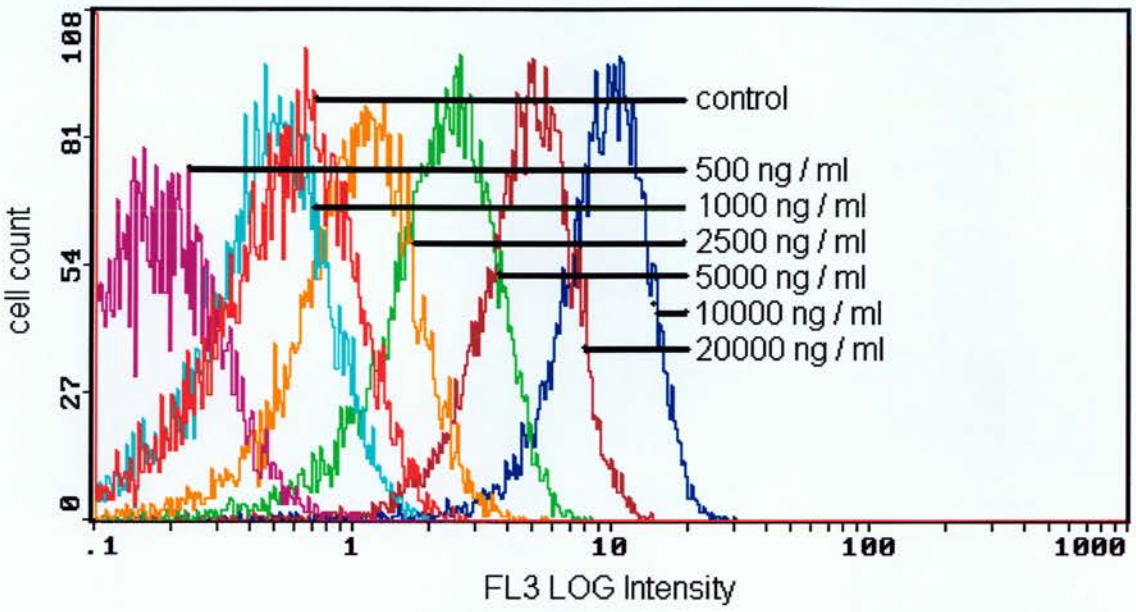
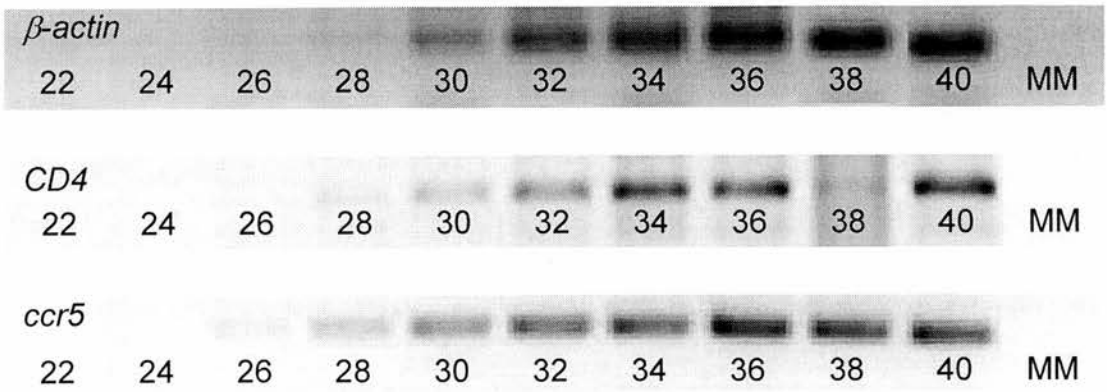
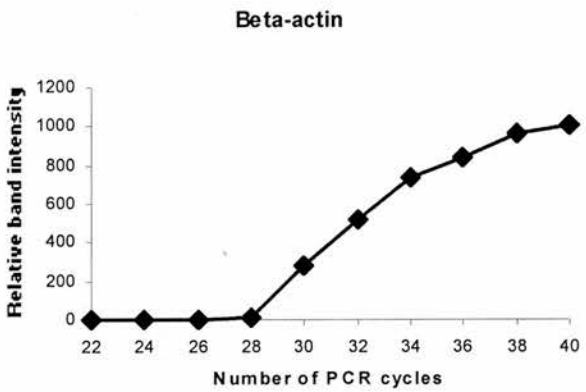
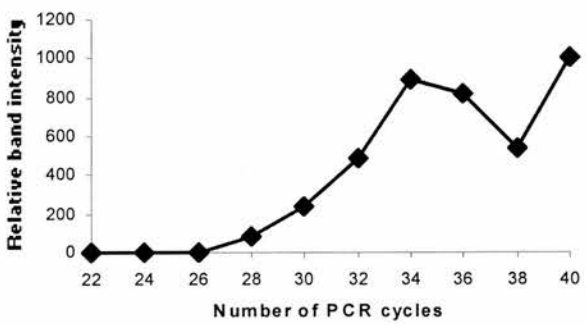


Figure 4.1. Anti-CD18 antibody titration. Anti-CD18 or an isotype control (at 20 μg ml^{-1}) was used to stain 10^5 macrophages at the concentrations shown. The R-PE conjugated secondary antibody was applied at a standard recommended concentration throughout. It can be seen that use of the primary antibody at a concentration of 10μg ml^{-1} gave the greatest staining intensity, this concentration was chosen for all subsequent experiments.


β-actin
22 24 26 28 30 32 34 36 38 40 MM
CD4
22 24 26 28 30 32 34 36 38 40 MM
ccr5
22 24 26 28 30 32 34 36 38 40 MM


Beta-actin
Relative band intensity
1200
1000
800
600
400
200
0
22 24 26 28 30 32 34 36 38 40
Number of PCR cycles


CD4
Relative band intensity
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1000
800
600
400
200
0
22 24 26 28 30 32 34 36 38 40
Number of PCR cycles

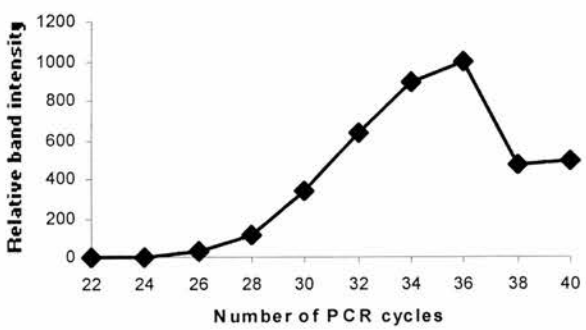

CCR-5
Relative band intensity
1200
1000
800
600
400
200
0
22 24 26 28 30 32 34 36 38 40
Number of PCR cycles

Figure 4.2 (previous page). Variable cycle RT-PCR. The electropherograms show PCR product bands obtained by amplification of an equal amount of starting material for the number of cycles indicated. Graphs showing corresponding band intensity values were produced by software analysis of the electropherograms. MM = 'master mix' (negative control).

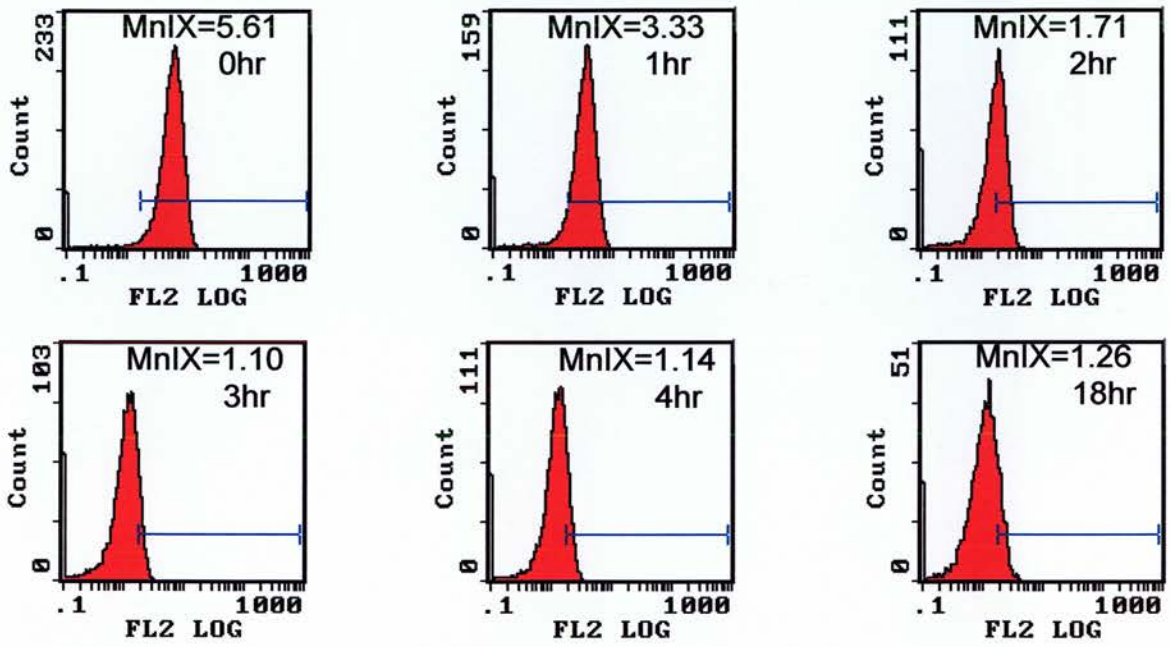


Figure 4.3. Loss of surface CD4. Flow cytometry histograms showing loss of surface CD4 from wildtype macrophages in response to incubation with R5-tropic gp120. The signal obtained from surface CD4 declined within one hour of incubation with R5-gp120 and reached its maximum extent by about 3 hours. The MnlX (mean fluorescence intensity) for isotype matched controls remained between 0.5 and 0.7 at all time points.

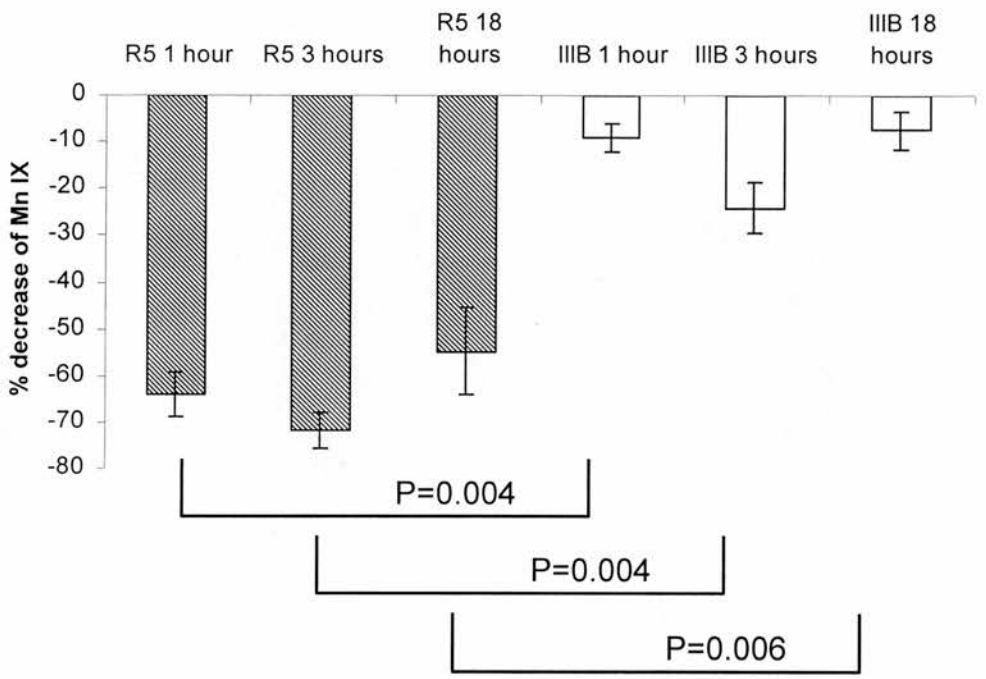


Figure 4.4. Surface CD4 levels. Macrophages were incubated with R5-tropic gp120 or $gp120_{IIIB}$ for the times indicated. The MnIX values, above the background fluorescence obtained with isotype control antibody staining, have been rescaled and expressed as a percentage decrease from the initial (untreated) staining intensity. Mean data values from experimental replicates with six different *ccr5Δ32* wildtype donors are shown. Bars show standard errors of the means. The P values were calculated using unpaired Mann-Whitney U tests.

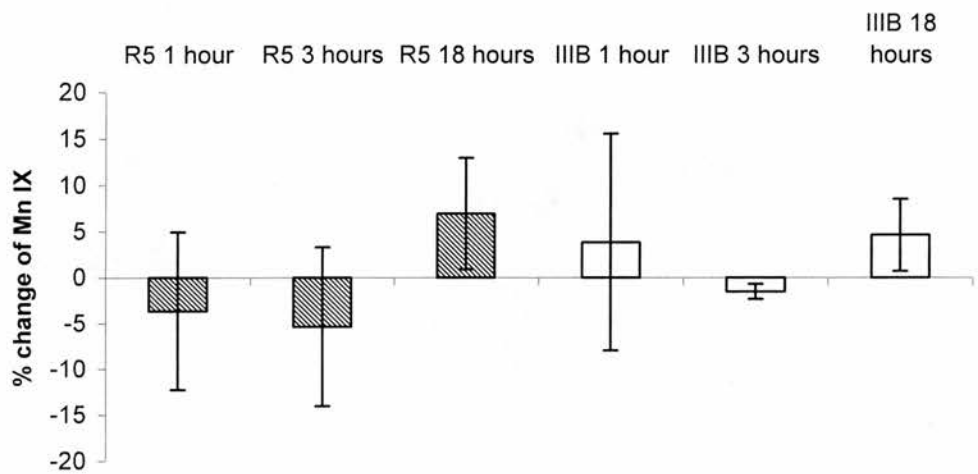


Figure 4.5. Surface CD4 levels. Macrophages were incubated with R5-tropic gp120 or $gp120_{IIIB}$ for the times indicated. The MnIX values, above the background fluorescence obtained with isotype control antibody staining, have been rescaled and expressed as a percentage change from the initial (untreated) staining intensity. Mean data values from experimental replicates with two different homozygous *ccr5Δ32* donors are shown. Bars show standard errors of the means.

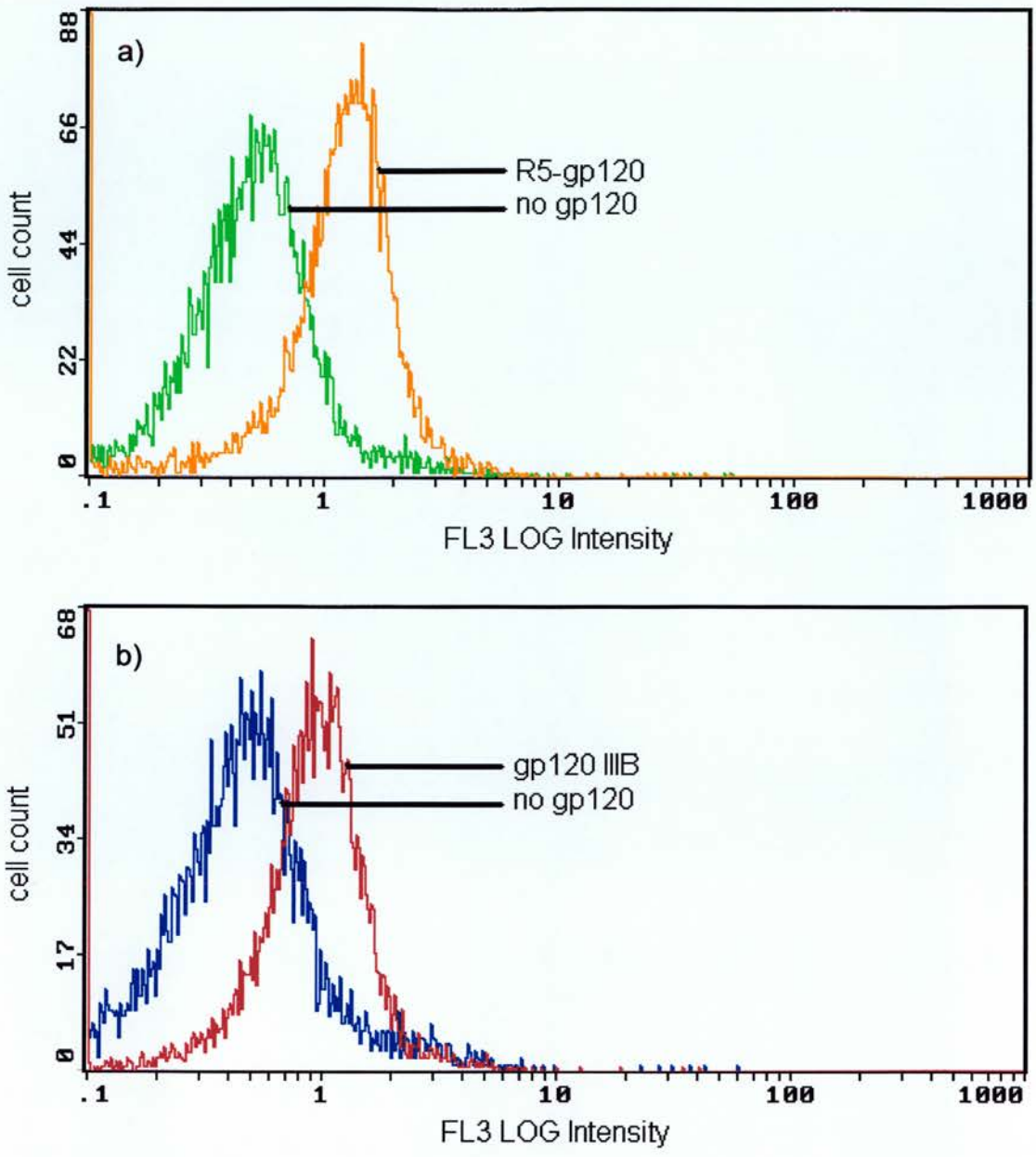


Figure 4.6. Detection of macrophage surface bound gp120. Macrophages were incubated with a) R5-tropic gp120 or b) $gp120_{IIIB}$. The surface bound gp120s were then detected by flow cytometry using an anti-gp120 polyclonal antibody, which was linked to FITC-conjugated strepavidin. Incubation of cells with gp120 resulted in a higher fluorescence signal than cells stained with anti-gp120 in the absence of gp120.

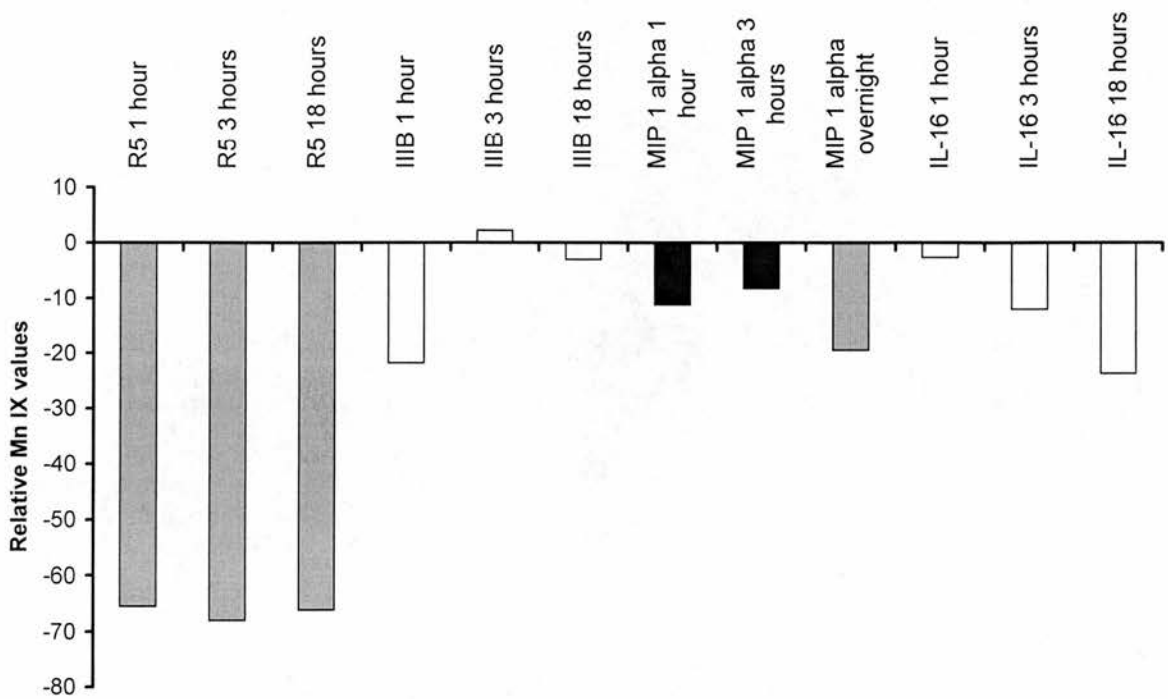
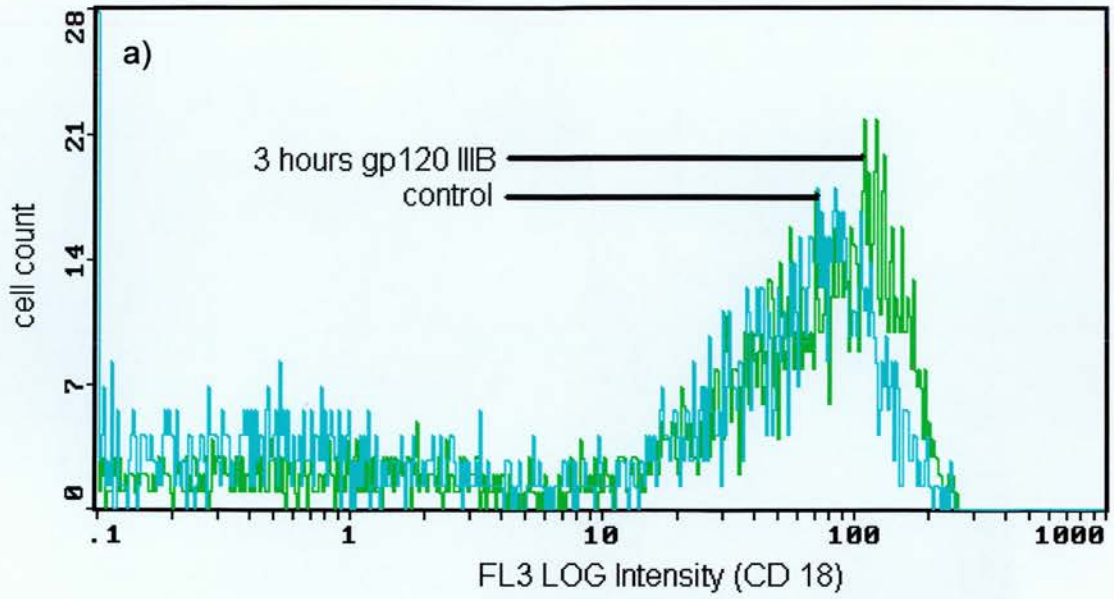
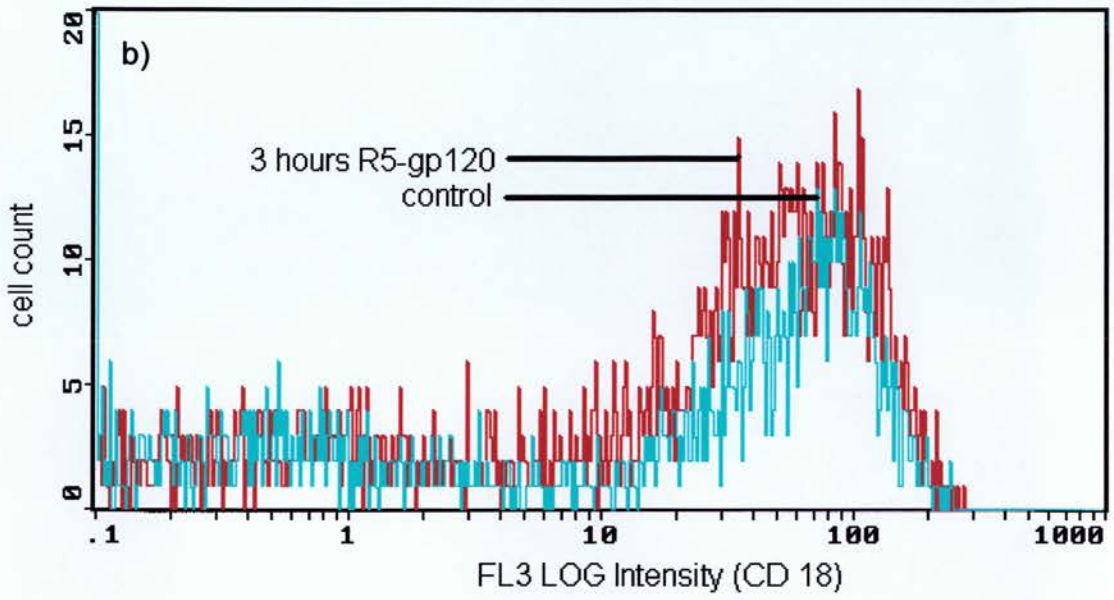


Figure 4.7. The effects of various CD4 or CCR-5 ligands on CD4 level. Macrophages from a single representative *ccr5Δ32* wildtype donor were incubated with R5-tropic gp120, $gp120_{IIIB}$, recombinant human IL-16 or recombinant human MIP-1α for the times indicated. The normalised MnIX values were calculated from the mean fluorescence intensities of the anti-CD4 labelled cells. The MnIX value, above the background fluorescence obtained with the isotype control, was then rescaled and expressed as a percentage change from the initial staining intensity.


a)
3 hours gp120 IIIB
control
cell count
0
7
14
21
28
.1
1
10
100
1000
FL3 LOG Intensity (CD 18)


b)
3 hours R5-gp120
control
cell count
0
5
10
15
20
.1
1
10
100
1000
FL3 LOG Intensity (CD 18)

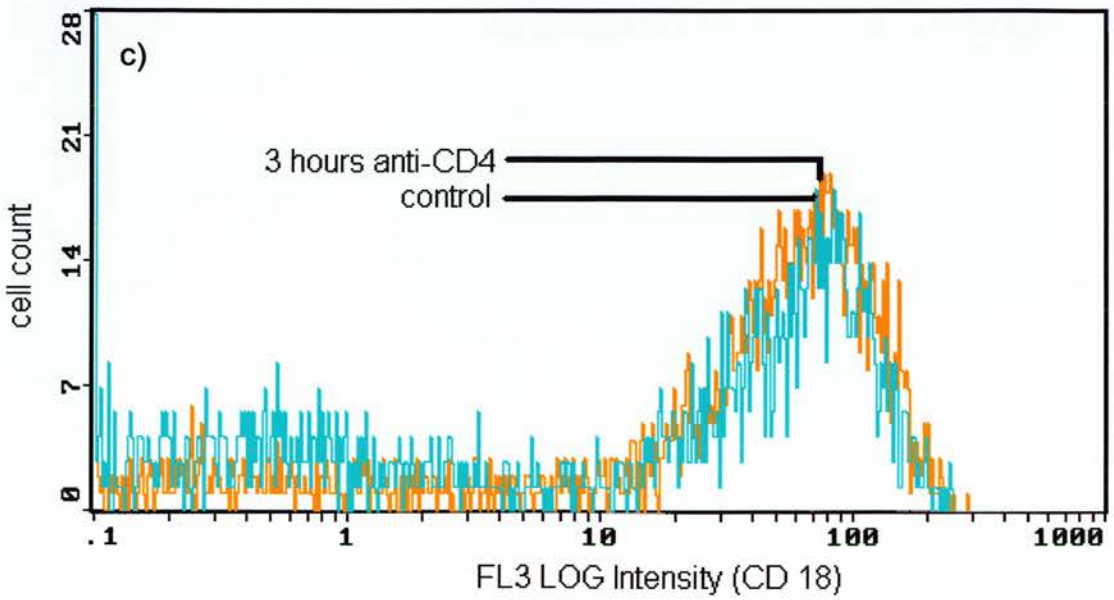

c)
3 hours anti-CD4
control
cell count
0
7
14
21
28
.1
1
10
100
1000
FL3 LOG Intensity (CD 18)

Figure 4.8 (previous page). Macrophage surface CD18 levels do not change. Monocyte-derived macrophages from a typical *ccr5Δ32* wildtype donor were incubated for 3 hours with a) $gp120_{IIIB}$, b) R5-tropic gp120 or a cross-linking anti-CD4 (QS4120) at concentrations previously demonstrated to induce a decline in surface CD4. Cells were then stained with anti-CD18 and showed similar CD18 levels by flow cytometry to those on control (untreated) cells.

Treatment	CD4 (MnIX)		CD18 (MnIX)	
	no anti-TNF	+ anti-TNF	no anti-TNF	+ anti-TNF
Nil – 0hr	11.1	12.5	61.0	45.4
$Gp120_{IIIB}$ – 1hr	7.93	8.06	58.5	43.8
$Gp120_{IIIB}$ – 3hr	8.63	8.68	52.0	44.1
$Gp120_{IIIB}$ – 18hr	8.49	8.75	N.D.	51.5
Nil – 0hr	11.1	12.5	61.0	45.4
R5-gp120 – 1hr	6.18	6.57	54.1	53.8
R5-gp120 – 3hr	4.61	4.63	64.4	49.6
R5-gp120 – 18hr	4.34	4.33	64.8	48.4
Nil – 0hr	11.1	12.5	61.0	45.4
Anti-CD4 – 1hr	4.38	4.43	66.7	55.1
Anti-CD4 – 3hr	4.12	3.80	62.2	55.3
Anti-CD4 – 18hr	4.32	4.10	70.7	N.D.

Table 4.1. Effect of TNF-α on macrophage CD4 loss. *Ccr5Δ32* wildtype macrophages were incubated with $gp120_{IIIB}$, R5-tropic gp120 or cross-linking anti-CD4 for the times indicated above, in the presence or absence of anti-TNF-α neutralising antibody, before having their surface CD4 and CD18 levels measured by flow cytometry. Surface antigen levels are expressed as mean fluorescence intensity values (MnIX).

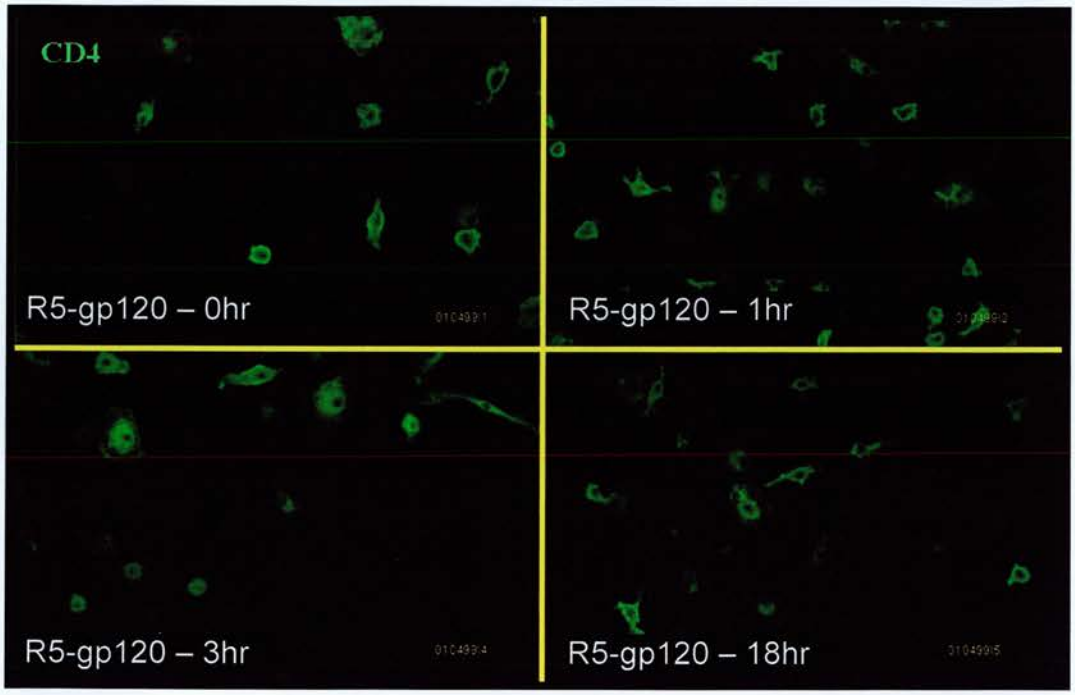
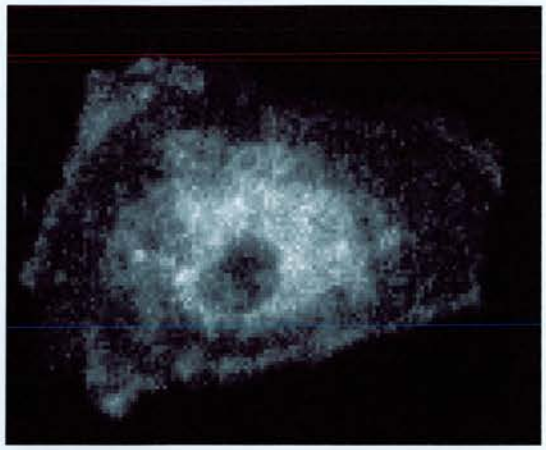
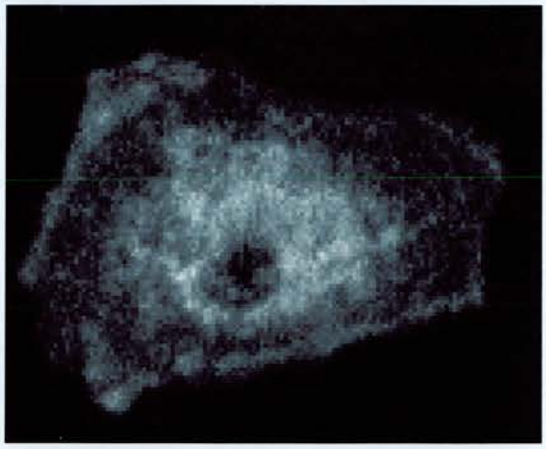


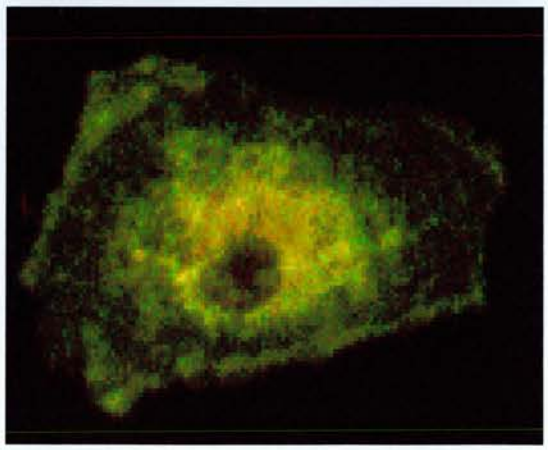
Figure 4.9. CD4 inside macrophages. Confocal micrographs of *ccr5Δ32* wildtype macrophages which have been incubated with R5-tropic gp120 for the times indicated, before being fixed, permeabilised and stained for CD4. At the zero time-point most of the CD4 can be seen on the cell surface. At the 3 and 18 hour time-points, there is less surface CD4 and more internal peri-nuclear CD4 visible.



a) MHC class II (HLA DR, TRITC)



b) CD4 (FITC)



c) overlay

MHC class II (red)

CD4 (green)

Figure 4.10. CD4 and MHC class II colocalise to an internal compartment. Confocal micrographs of a *ccr5Δ32* wildtype macrophage, which has been incubated with R5-tropic gp120 for 3 hours and then fixed, permeabilised and stained red for MHC class II (HLA DR, figure 4.10a) and green for CD4 (figure 4.10b). CD4 and MHCII are colocalised both at the cell surface and intracellularly, as indicated by the coincidence of red and green stains to give a yellow image (figure 4.10c).

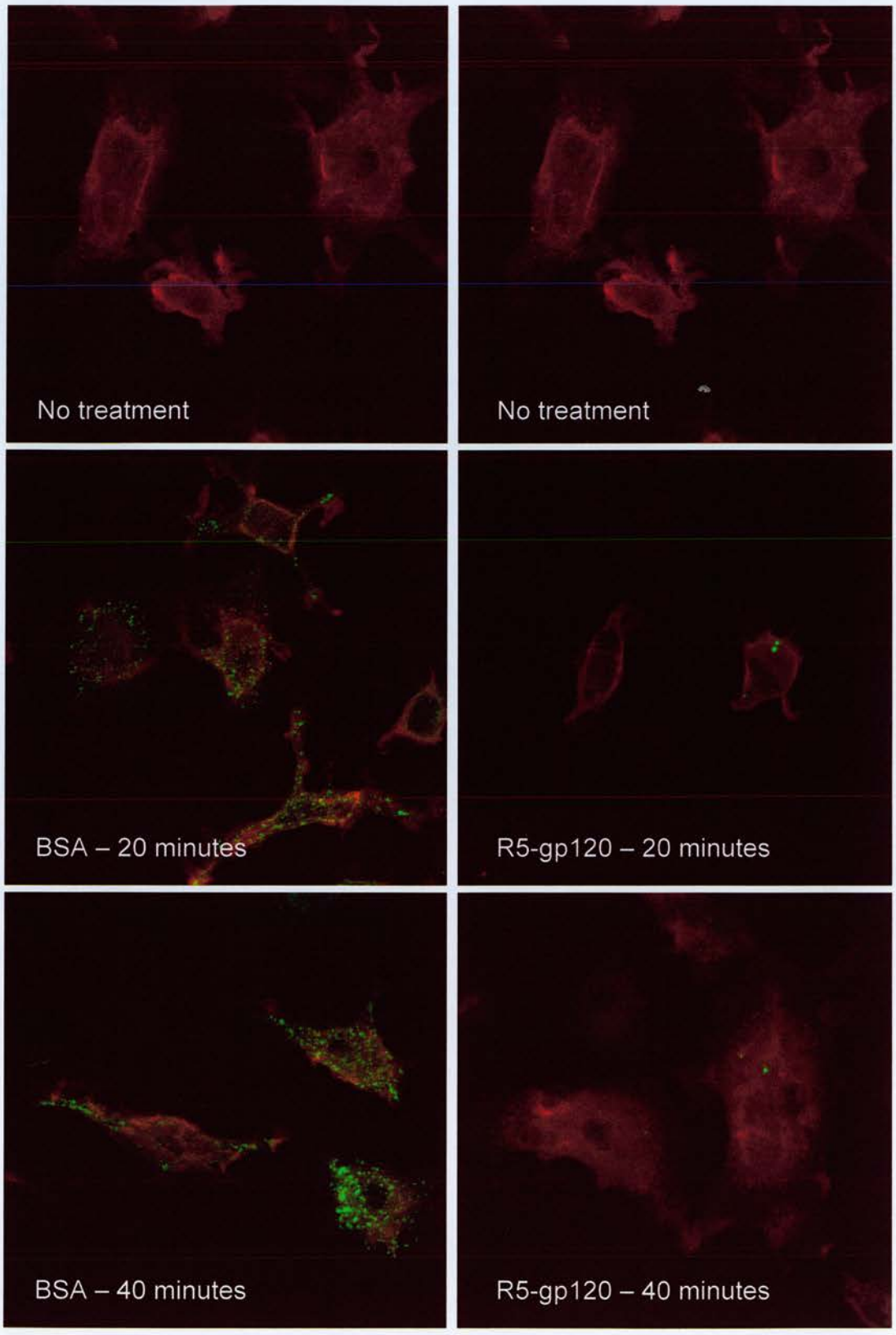


Figure 4.11 (continued over).

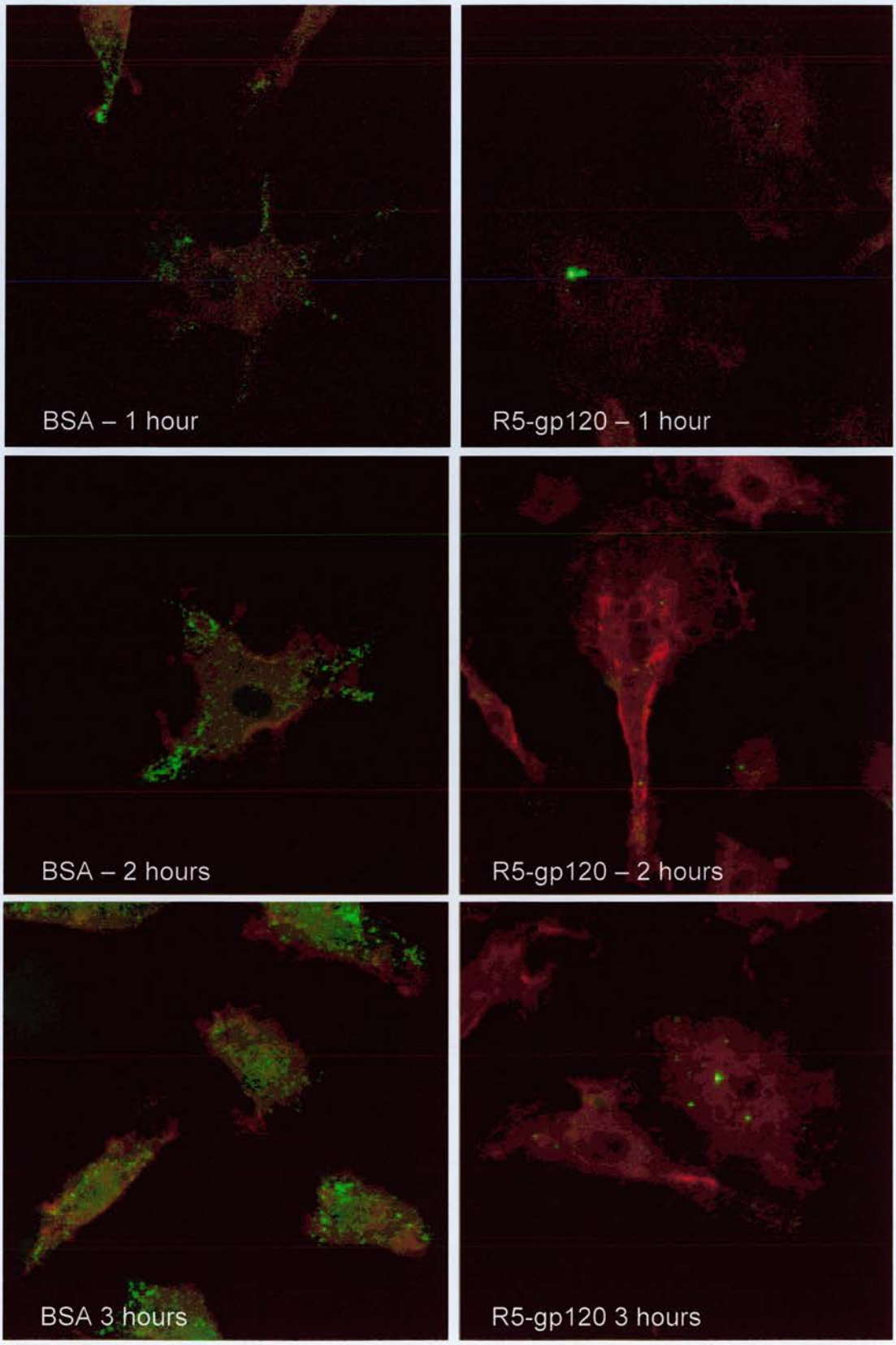


Figure 4.11 (continued over).

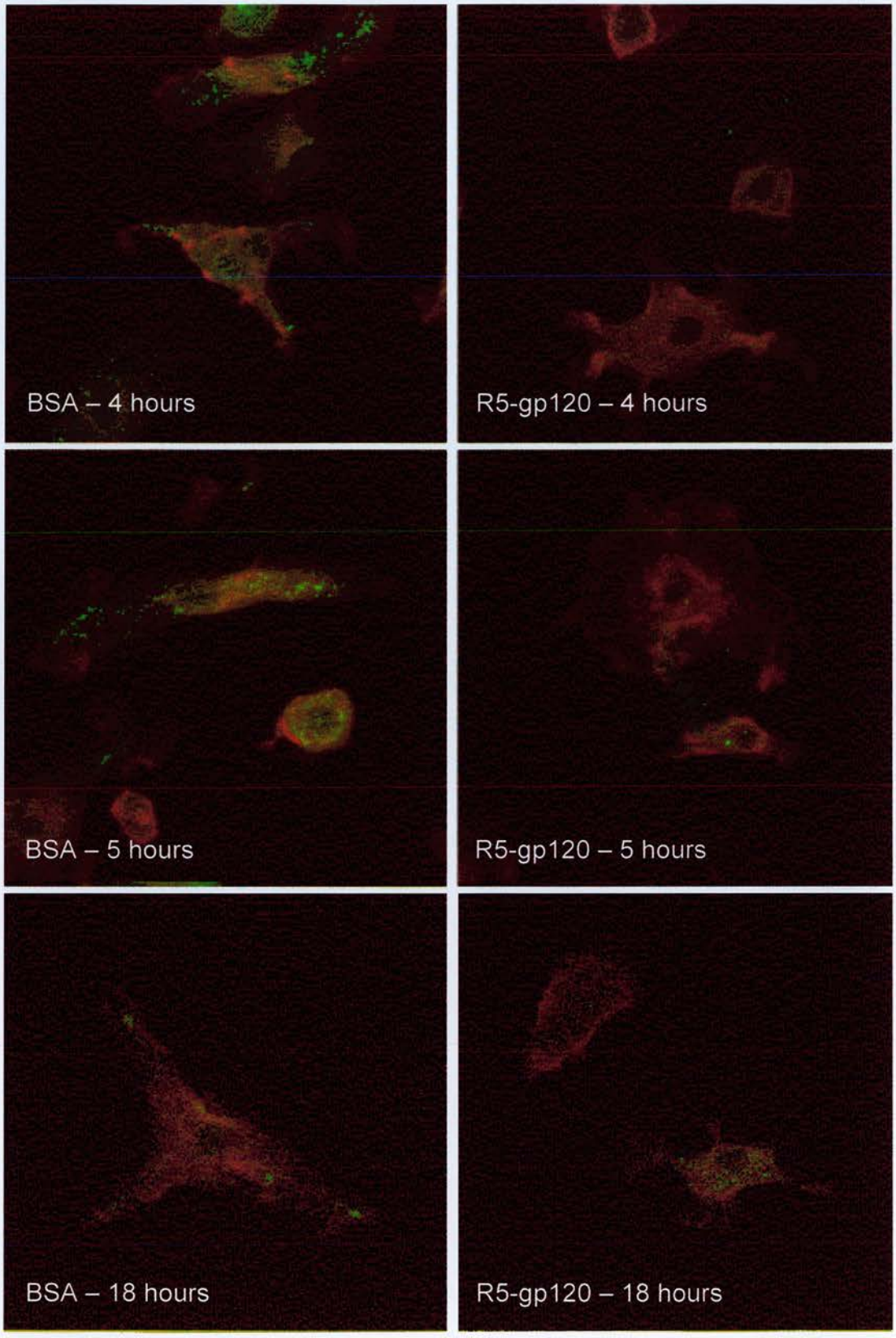


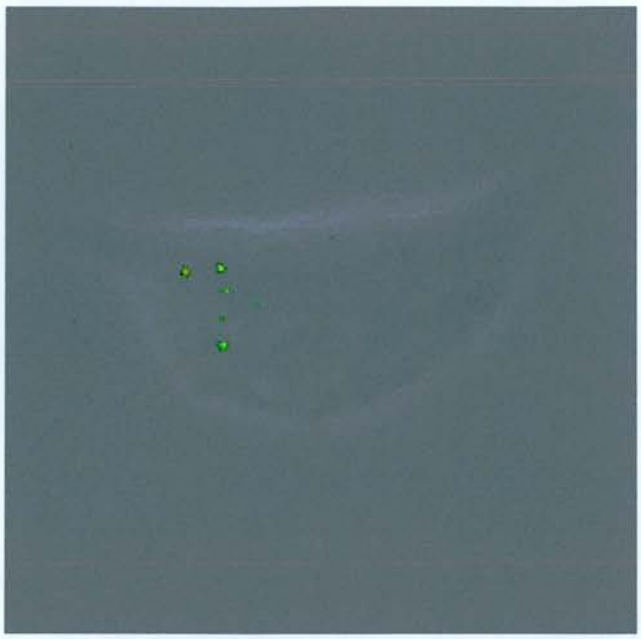
Figure 4.11 (caption over page).

Figure 4.11 (previous page). Internalised gp120 and BSA. *Ccr5Δ32* wildtype macrophages were incubated with FITC-conjugated R5-tropic gp120 or FITC-conjugated BSA (as a control) for the times indicated, then fixed, permeabilised and stained red for HLA DR. Internalised protein is visible in green. Colocalisation of green and red stains is shown as yellow.

Time	FITC-BSA staining				FITC-R5-gp120 staining			
	large bright dots?	small dots?	colocali-sed with HLA DR ?	diffuse staining ?	large bright dots?	small dots?	colocali-sed with HLA DR ?	diffuse staining ?
start	-	-	N/A	N/A	-	-	N/A	N/A
20min	-	+	-	-	+	-	-	-
40min	-	+	-	-	+	-	-	-
1hr	-	+	-	+/-	+	+	-	-
2hr	-	+	-	+	+	+	+	+/-
3hr	-	+	+/-	+	+	+	-	+
4hr	-	+	+	+	-	+	-	+
5hr	-	+	-	+	-	+	-	+
18hr	-	+/-	-	+	-	+	-	+

Table 4.2. Endocytosis characteristics. *Ccr5Δ32* wildtype macrophages were incubated with FITC conjugated R5-tropic gp120 or BSA for the times indicated before being fixed, permeabilised and stained for HLA DR. The table summarises the characteristics of endocytosed FITC-conjugated protein in different cell populations. Specimens were scored for the presence of endocytosed protein giving the appearance of either large or small intracellular dots, or a diffuse cytoplasmic haze. Colocalisation of endocytosed protein with MHC class II (HLA DR) was also noted.

-, not present; +/-, present in some cells; +, present in most cells; N/A, not applicable.



a) FITC-anti-mouse antiserum – 1 hour

b) pre-incubated complex of (FITC-anti-mouse antiserum / mouse anti-CD4) – 1 hour

Figure 4.12. Pinocytosis compared with receptor mediated phagocytosis. Phase contrast micrographs of macrophages superimposed with confocal immunofluorescence images (green). Figure 4.12a shows pinocytic vesicles formed after a 1 hour incubation with FITC conjugated immunoglobulins, which do not have cell surface ligands. Figure 4.12b shows a large phagocytic vesicle formed by a 1 hour incubation with FITC-conjugated immunoglobulins which have been pre-complexed to anti-human CD4, allowing the complexes to recognise the CD4 macrophage surface antigen.

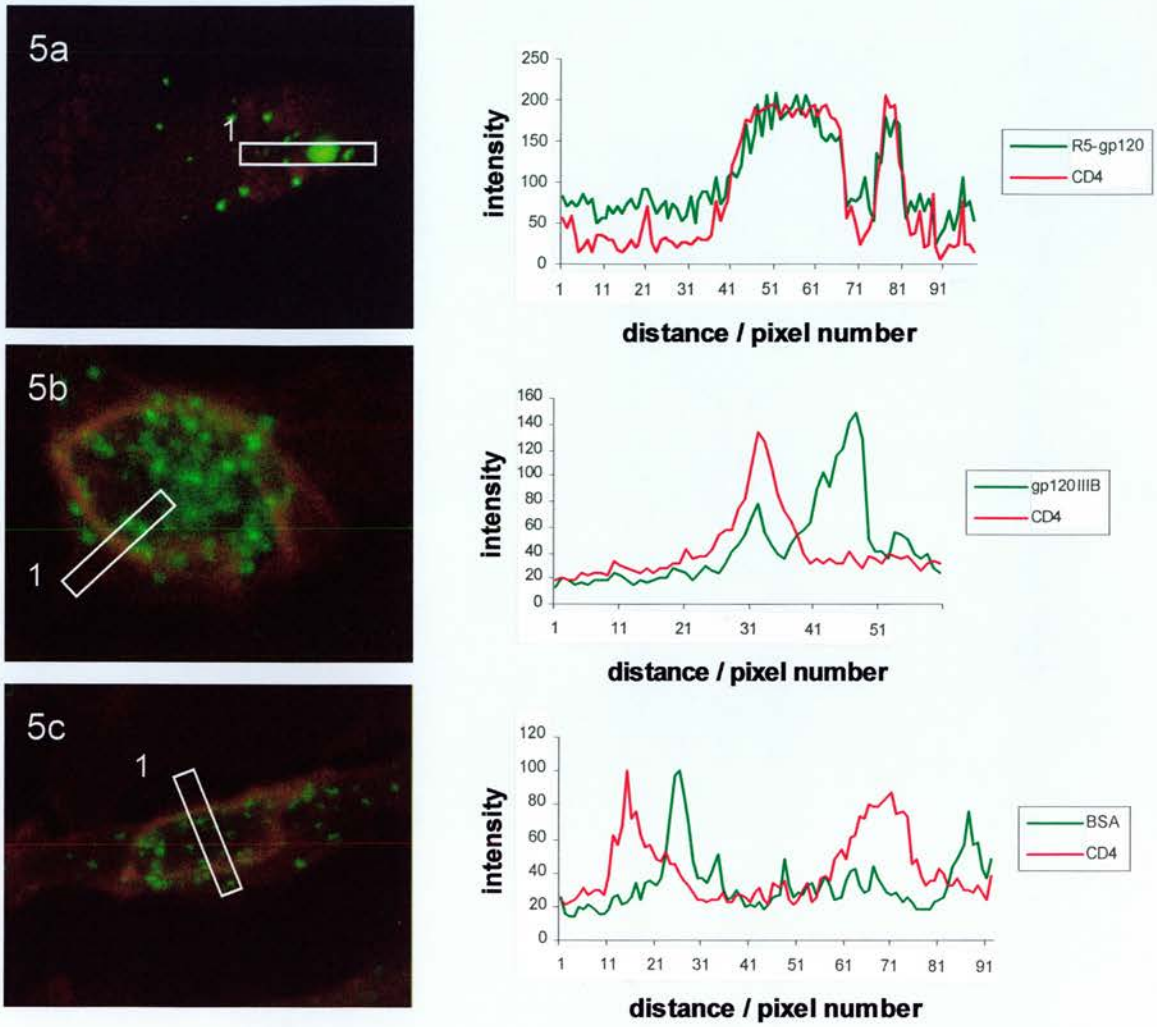


Figure 4.13. Analysis of endocytosed protein. Confocal micrographs show *ccr5Δ32* wildtype macrophages which have been incubated with FITC-conjugated R5-tropic gp120 (figure 4.13a), FITC-gp120$_{IIIB}$ (figure 4.13b), or a control FITC-conjugated protein (BSA, figure 4.13c) for 20 minutes before being fixed, permeabilised and stained red for CD4. Internalised protein is visable in green. Coincidence of red and green labelling is visible as yellow. The coincidence of endocytosed protein and CD4 in figure 4.11a but not 4.11b or 4.11c was confirmed by software analysis of the images and shown by the resultant intensity plots, which show the intensity of red and green labelling along a line drawn through the images as indicted.

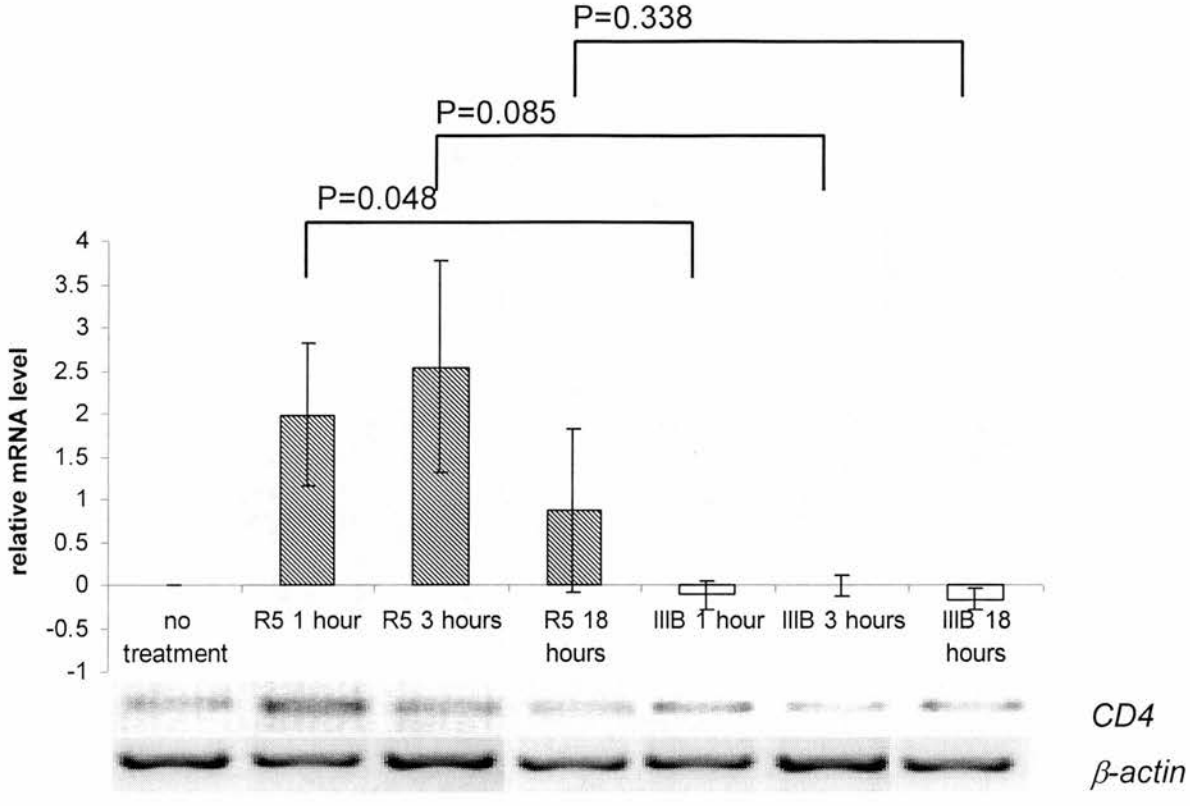


Figure 4.14. CD4 mRNA levels. The graph shows the ratio of *CD4* to *β-actin* gene transcripts in RNA extracted from *ccr5Δ32* wildtype macrophages, pre-treated with R5-tropic gp120 or $gp120_{IIIB}$ for the times indicated, as determined by semi-quantitative RT-PCR. Ratios are expressed as changes from the ratio obtained for the no-treatment controls. The graph shows the mean values obtained from six different donors. The bars show standard error of the means. The P values were calculated using unpaired Mann-Whitney U tests. The PCR gel picture beneath the graph shows typical raw data obtained from one of the six donors.

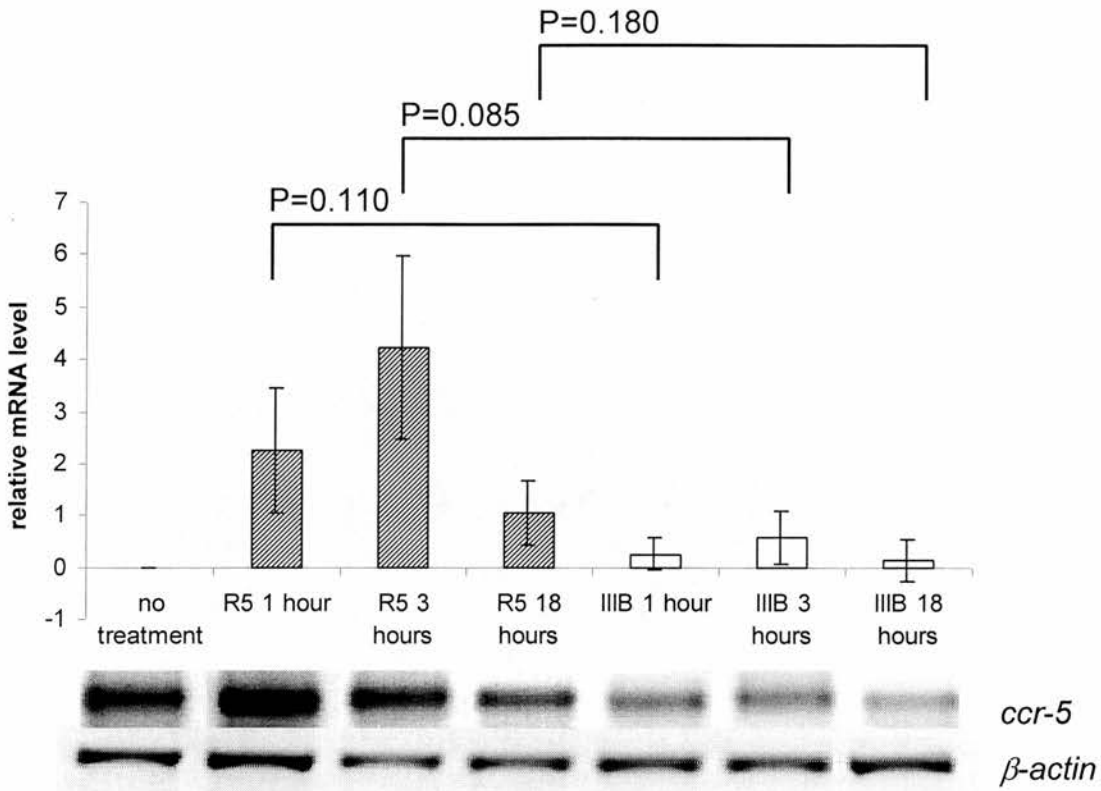


Figure 4.15. CCR-5 mRNA levels. The graph shows the ratio of *ccr-5* to *β-actin* gene transcripts in RNA extracted from *ccr5Δ32* wildtype macrophages, pre-treated with R5-tropic gp120 or $gp120_{IIIB}$ for the times indicated, as determined by semi-quantitative RT-PCR. Ratios are expressed as changes from the ratio obtained for the no-treatment controls. The graph shows the mean values obtained from six different donors. The bars show standard error of the means. The P values were calculated using unpaired Mann-Whitney U tests. The PCR gel picture beneath the graph shows typical raw data obtained from one of the six donors.

Discussion

It has been reported that HIV-1 gp120 is able to disrupt the function of immune cells (Fanci, 1995; Rodriguez and Hard, 1995; Hewson *et al.*, 1999) and one of its actions is to cause a loss of cell-surface CD4 and the functions associated with this protein (Wahl *et al.*, 1989; Theodore *et al.*, 1994; DurrbaumLandmann *et al.*, 1994; Karsten *et al.*, 1996). This thesis identifies a novel, CCR-5-dependent mechanism of CD4 decline. A relatively small (about 25%) surface CD4 decline was induced on macrophages by $gp120_{IIIB}$ (figure 4.4), although this effect did not reach statistical significance, largely due to the high degree of variability between donors. This X4-tropic $gp120_{IIIB}$-induced response may be similar to the CD4 loss reported previously (Wahl *et al.*, 1989; Karsten *et al.*, 1996), although the observation that neutralisation of TNF-α did not abrogate $gp120_{IIIB}$-induced CD4 loss contradicts the findings of Karsten *et al.*, 1996.

Although CXCR-4 is expressed on human macrophages and can, under some circumstances, be utilised for HIV-1 entry by dual-tropic HIV-1 strains (Yi *et al.*, 1999), it appears to be unusable and possibly inaccessible to many T-cell line adapted X4-tropic gp120s including $gp120_{IIIB}$ which was used in this study as a prototypic X4-tropic gp120. It is therefore proposed that $gp120_{IIIB}$ is only able to interact with macrophages via CD4. Figure 4.6 presents evidence that $gp120_{IIIB}$ was able to bind to the macrophage surface, presumably this interaction was wholly via CD4.

When R5-tropic gp120 was incubated with macrophages, a cell-surface CD4 loss that was significantly more substantial than both previous reports of gp120-induced CD4 loss and our observations with X4-tropic $gp120_{IIIB}$ resulted. Approximately 75% of the surface CD4 was lost by 3 hrs after addition of gp120 (figure 4.4). This observation may require a novel mechanism of CD4 loss to be proposed. Further evidence implicates CCR-5 binding, in addition to CD4 binding as a requirement for the operation of this novel mechanism.

The requirement for gp120 to bind to CCR-5 in order to obtain a substantial CD4 loss is suggested by the strain specificity of the effect, with substantial loss only

observed when R5-tropic gp120 is used. Further evidence for a CCR-5 binding requirement comes from the observation that CD4 loss is not observed in mutant macrophages, which do not express CCR-5 (figure 4.5). Binding to CD4 only (by $gp120_{IIIB}$, IL-16 or by R5-tropic gp120 on *ccr5* null cells) or binding to CCR-5 only (by MIP-1α) does not induce substantial CD4 loss (figure 4.7). This observation supports earlier reports that recombinant IL-16 (Theodore *et al.*, 1996) or transfection with IL-16 cDNA (Zhou *et al.*, 1997) fail to cause a surface CD4 decline from T-cells. However, binding of surface CD4 only by an anti-CD4 antibody results in a substantial surface CD4 loss resembling that induced by R5-tropic gp120 (table 4.1). The crucial difference between anti-CD4 and R5-tropic gp120, and IL-16, MIP-1α and $gp120_{IIIB}$ may be that the first two proteins are able to cause enough cross-linking of cell surface antigens for patching, capping and receptor mediated phagocytosis to result.

R5-tropic gp120-induced CD4 loss by this newly described mechanism, therefore requires gp120 to bind to both CD4 and CCR-5. We propose that the CD4 loss observed be due to cross-linking of CD4 and CCR-5 on the macrophage cell surface followed by endocytosis of the tri-protein complex (figure 4.16). Single ligation of CD4 or CCR-5 does not produce sufficient cross-linking to allow endocytosis and CD4 loss by this mechanism. An assumption of this model is that it is the same molecule of gp120 that binds to both cell-surface receptors. CXCR-4 on the macrophage appears to be inaccessible to X4-tropic $gp120_{IIIB}$ binding in this way, whereas CCR-5 is present on the macrophage cell surface in a form that allows R5-tropic gp120 to bind to both it and CD4 (Yi *et al.*, 1999). It may be that CD4 and CCR-5 are pre-associated in some way on the macrophage cell surface and that this allows single molecules of R5-tropic gp120 to bind to these surface proteins and produce the CD4 loss demonstrated. Such a pre-association could allow CCR-5 to mediate HIV-1 infection of APCs more efficiently and explain the apparent selection by HIV-1 of CCR-5 for almost exclusive use as a co-receptor for macrophage infection. If CD4 loss were indeed the result of phagocytosis of tri-protein complexes, one would expect to see a loss of macrophage surface CCR-5 concomitant to the CD4 loss. Attempts to show this were frustrated by the lack of anti-CCR-5 antibodies able to stain CCR-5 brightly enough to allow semi-

quantitative estimation of receptor densities. The observation that *ccr5* mRNA is up-regulated following CD4 loss (figure 4.15) does however provide indirect evidence for earlier loss of surface CCR-5.

The analogous *in vivo* situation may be more complicated. Gp120 has been found at high levels in the serum of AIDS and ARC patients (Oh *et al.*, 1992). Serum antibodies to gp120 may increase the extent of macrophage surface receptor cross-linking, bring the Fc receptor into play, and reduce the CCR-5-binding requirement. It has been observed that most of the gp120 found in human serum is in the form of immune complexes (Oh *et al.*, 1992) and that antibodies to gp120 can enhance cell infection by increasing virion-to-cell binding (Toth *et al.*, 1994).

Our model of cross-linking-induced endocytosis is strengthened by the confocal images obtained. Figures 4.11 and 4.13 show that R5-tropic gp120 enters macrophages in a form that is different, in terms of size and number of vesicle and localisation with CD4, from pinocytosis of BSA, a protein with no specific cell-surface receptor and endocytosis of $gp120_{IIIB}$, a protein which is unable to use both a cell-surface receptor and a co-receptor. R5-tropic gp120 enters cells as a few large vesicles, possibly by a process of patching and capping. Endocytosed $gp120_{IIIB}$ and BSA appear in macrophages in a form which suggests that they entered by a process more akin to constitutive pinocytosis (Cohn and Steinman, 1982). Endocytosis of R5-tropic gp120 appeared very similar to the entry of cross-linked anti-CD4 (figure 4.12b), and entry of BSA and $gp120_{IIIB}$ resembles the pinocytosis of protein with no cell surface receptor (figure 4.12a). The presentation efficiency of endocytosed protein to $CD4^+$ and $CD8^+$ T-cells was not investigated, nor was the context, in terms of the cytokine environment and co-stimulatory signals. However, endocytosed CD4 (figures 4.11 and 4.13), newly synthesised CD4 (figure 4.10) and endocytosed R5-gp120 and endocytosed BSA (figure 4.11) were all detected in MHC class II containing cellular compartments making presentation of these proteins a possibility.

If our proposed model of cross-linking induced endocytosis operates *in vivo*, there are implications for processing and presentation of large amounts of both viral and self-protein entering an antigen presenting cell. The induction of protective and autoimmune responses could be influenced if the endocytosed proteins were finding

their way into antigen presentation pathways and being presented with unusually high efficiency.

After R5-tropic gp120-induced cell surface loss of CD4 (and presumably CCR-5 also) the cellular production of these proteins is stepped up. This is manifested as an up-regulation of mRNA transcript (figures 4.14 and 4.15), and at least in the case of CD4 at the level of increased translation of mRNA into intracellular pools of protein (figure 4.9). Increased levels of intracellular self-protein must have *in vivo* implications for the possible breaking of tolerance and the induction of auto-immune responses to these proteins. There are reports of self tolerance to CD4 (Chams *et al.*, 1988; Caporossi *et al.*, 1998) and other self antigens (Magnac *et al.*, 1990; Marchalonis *et al.*, 1997; Stricker *et al.*, 1998) being lost in HIV-1 infection. There is also potential for immune dysregulation when large amounts of viral (gp120) and self (CD4 / CCR-5) antigen are presented by the same APC. If the immune system is already tolerant of CD4 and CCR-5, this may be due to the presence of regulatory T-cells specific to these antigens. Bystander suppression (discussed in chapter 5) could allow tolerance to CD4 and CCR-5 to spread to the foreign antigen gp120.

The work discussed in this chapter also has implications for therapeutics (such as those discussed in chapter 5) designed to block infection by binding to CD4 and CCR-5. It may be that agents which cause too much cell-surface receptor cross-linking should be avoided because of their potential to produce immune system dysregulation.

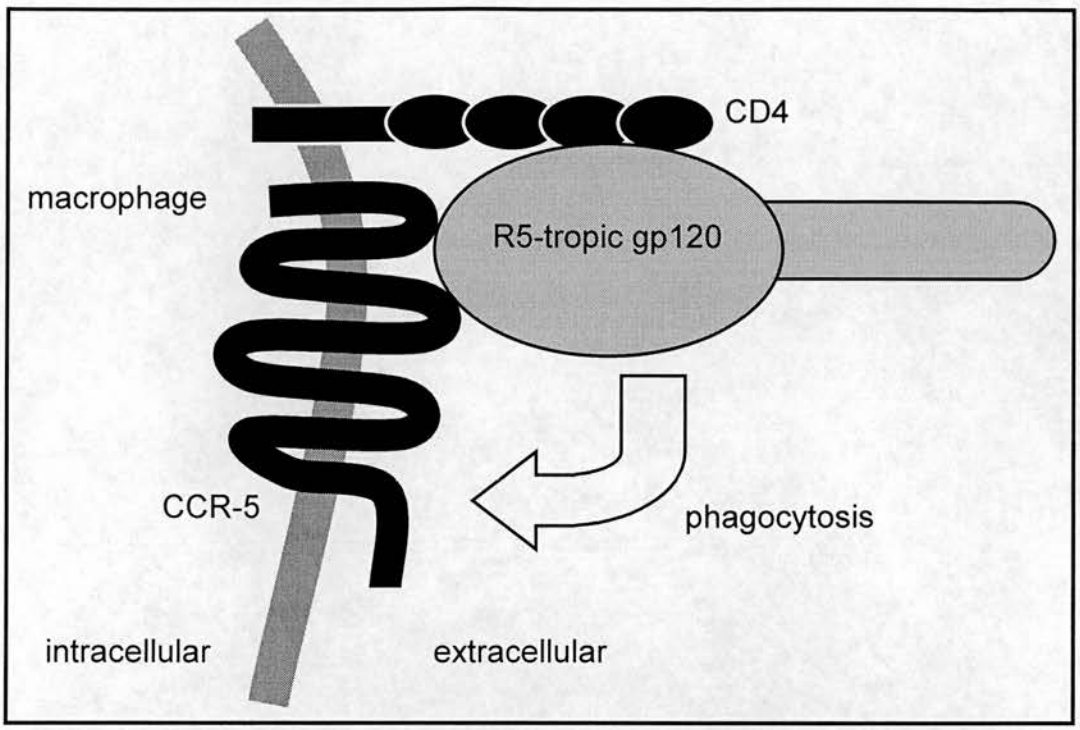


Figure 4.16. Hypothesis for surface CD4 loss. The observation that substantial and rapid gp120-induced loss of CD4 is dependent on gp120 being able to bind to both cell-surface CD4 and CCR-5 suggests that the CD4 loss may be a result of R5-tropic gp120 causing sufficient cross-linking of membrane components for the cross-linked complexes to be internalised by receptor mediated phagocytosis.

CHAPTER 5: RESULTS

HIV-1, TOLERANCE AND NOTCH

N.B. *Many of the genes and proteins described in this chapter have multiple names and different names in different species. To reduce confusion, the same name will be used here regardless of the species. A list of names and their equivalents is given at the start of this thesis.*

Background

This chapter explores the nature of immunologic tolerance and speculates as to how the mechanisms of tolerance might be subverted by HIV-1 in order to cause disease. It will then explain how the Notch signalling pathway, important in embryology, might be involved in the induction of tolerance. Data showing how HIV-1 gp120 interacts with the Notch pathway will then be presented and discussed. The central hypothesis tested is that HIV-1 gp120 manipulates the Notch signalling pathway in order to induce inappropriate 'tolerance' which contributes to the immune deficiency seen in AIDS.

Tolerance

One definition of *tolerance* is a state of non-responsiveness in the face of provocation that *ought* to elicit a response. *Immunologic tolerance* can be defined as a state of non-responsiveness to an antigen that *ought* to elicit a response. Just as it can be argued that an individual can not be truly tolerant of something of which he is ignorant, immunologic tolerance differs from immune-ignorance, which is non-responsiveness to an antigen that the immune system simply has not encountered (Chen, 1998a; Chen, 1998b; Hausmann *et al.*, 1999). Tolerance of self-antigen is a central feature of the immune system. Self-antigen *ought* to elicit a response; indeed, if the antigen is transferred to another individual (as in a graft), a response does result.

Working definitions of tolerance are often along the lines of "tolerance is a failure to elicit the type of response that we happen to be measuring in our experiments." If one is only measuring antibody responses, a cell-mediated response might be missed and mistaken for tolerance. Similarly, the failure of an antigen to elicit a Th1-type cytokine response may be interpreted as immunologic tolerance of the antigen, or as an *immune-deviation* from a Th1 to a Th2-type response (Kuribayashi *et al.*, 1997; Ekerfelt *et al.*, 1999). Defining tolerance as a state of non-responsiveness implies that it is a passive process – simply the lack of the active processes of immune-activation. In many situations however, tolerance is actively mediated, whether by the induction of apoptosis, anergy, or suppressor / regulatory cells (Wood, 1996). As such tolerance is not truly *unresponsiveness*, rather it is *different-responsiveness*.

Danger

The danger hypothesis (Matzinger, 1994; Pennisi, 1996) states that the function of the immune system is to discriminate between dangerous and safe antigens, rather than self and non-self antigens. It proposes that the immune system perceives danger signals from microbial components and (necrotic) tissue damage and will only mount a response to antigen encountered in this context. The immune system tolerates antigens encountered in the absence of a danger signal. The APC is proposed to play a central role in receiving danger signals (Matzinger, 1994; Ridge *et al.*, 1996). The danger hypothesis is attractive because it explains how in the absence of complete deletion of autoreactive lymphocytes (Genain *et al.*, 1994) the immune system is tolerant of self-antigens encountered in the absence of danger signals. The danger hypothesis is less successful at explaining autoimmunity; why do lymphocytes start to attack self-tissue in the apparent absence of danger signals? One explanation for this is that a transient infection provides the danger signal required to initiate autoimmunity. This begs the question: how can autoimmune disease be induced experimentally (Aabakken and Osnes, 1989; Metzler and Wraith, 1993; Powrie, 1999) in the absence of infection? The fact that autoimmune responses to self antigen can cause disease in some individuals stresses the importance of active self tolerance in the normal homeostatic regulation of the immune system.

Mechanisms of T-cell tolerance

Thymic selection. Not all newly generated thymocytes reach the periphery; many are deleted by apoptosis in the thymus (Kruisbeek and Amsen, 1996). As well as the deletion of developing T-cells that do not express a TCR able to recognise peptides held in self-MHC molecules, T-cells with specificity for self-peptides, or which bind too tightly to self-MHC are deleted (Janeway, 1994; Jameson and Bevan, 1995; Kishimoto and Sprent, 2000). IL-4 and IL-7 can inhibit this thymocyte apoptosis (Kishimoto and Sprent, 1999), and mice which lack pro-apoptotic genes (for Fas and Fas Ligand), or over-express anti-apoptotic genes (*bcl2*) develop autoimmune disease (Strasser *et al.*, 1991; Suda and Nagata, 1997; Weintraub *et al.*, 1998; Weintraub and Cohen, 1999). Self-peptide / MHC complexes that are absent from the thymus, or which are present at low density, are unable to trigger the death of T-cells which can recognise them. These potentially self-reactive T-cells can then escape to the periphery (Liu *et al.*, 1995; Kawai and Ohashi, 1995).

Peripheral tolerance. Self-reactive T-cells that encounter self-antigen in the periphery can be induced to undergo apoptosis or become anergic. A variety of mechanisms can induce this peripheral tolerance. Immune privileged sites such as the retina, the anterior chamber of the eye, and the testes may express Fas ligand (FasL, CD140), which can engage Fas (CD95) on T-cells to give them a death signal (Griffith *et al.*, 1995; Bellgrau *et al.*, 1995; Ferguson and Griffith, 1996a; Ferguson *et al.*, 1996b; Stuart *et al.*, 1997). Recently, however, some doubt about a general role for Fas / FasL interactions in immune privilege has emerged because of the small number of good studies undertaken and the reliability of reagents (Restifo, 2000). Chronic stimulation of the TCR, especially with high-dose antigen can favour elimination (Sprent and Webb, 1995; Renno *et al.*, 1995) or silencing (Dillon *et al.*, 1995; Rocha *et al.*, 1995) of T-cells. Activated cells can also become primed for Fas or TNF mediated activation induced cell death (AICD, Zheng *et al.*, 1995; Bonfoco *et al.*, 1998; Gao *et al.*, 1998). T-cells may be deleted if they receive a signal through their TCR at the same time as CTLA-4 engagement by CD28 (Krummel and Allison, 1995; Krummel *et al.*, 1996; Punt *et al.*, 1997). If a T-cell encounters antigen on a parenchymal cell which is not expressing B7 or an equivalent costimulatory molecule (only professional APCs express B7 in a non-inflamed site) it will ignore

the antigen and become anergic (Mueller and Jenkins, 1995; Boise *et al.*, 1995; MarelliBerg and Lechler, 1999).

Immune deviation. Whether or not immune deviation (the polarisation of a response towards one that is Th1 or Th2 dominated) can be described as a form of tolerance is largely a semantic argument. Although at a cellular level T-cells are persistently active, immune deviation can produce *functional tolerance* at the level of the organism. In some animal models of autoimmune diseases such as the NOD mouse, the tissue of non-diseased animals is extensively infiltrated by activated T-cells. This T-cell response, however, has been skewed to a lineage which does not mediate disease (Finkelman, 1995; Katz *et al.*, 1995; Liblau *et al.*, 1995; Liblau *et al.*, 1997). The control of immune deviation and the development of Th-cell subsets is complicated, but APCs are thought to play an important role by providing an appropriate environment of cytokines and other signals for T-cell subset development (Kuchroo *et al.*, 1995; Finkelman, 1995; Carter *et al.*, 1998).

Regulatory / suppressor T-cells. Despite initial controversy over the existence of regulatory / suppressor T-cells (Moller, 1988), there is mounting evidence that a subclass of thymus derived, $CD4^+$ T-cells can provide antigen-specific protection against the potentially damaging effects of other activated T-cells (Groux and Powrie, 1999; Shevach, 2000). A role for regulatory T cells (Tr-cells) has been demonstrated in several animal models of autoimmunity, including inflammatory bowel disease (IBD, Groux and Powrie, 1999) and experimental autoimmune encephalitis (EAE, Chen *et al.*, 1994; Kumar and Sercarz, 1998). If animals are depleted of these regulatory cells autoimmune disease develops. Adoptive transfer of regulatory T-cells can inhibit the disease. The regulatory T-cells that provide protection from IBD by suppressing T-cells activated against the bacterial flora of a healthy gut are $CD4^+$, $CD45RB^{lo}$ and are enriched within the $CD25^+$ subset (Groux and Powrie, 1999; Powrie, 1999). $CD4^+$, $CD45RB^{lo}$ T-cells from IL-10 knockout mice fail to protect from IBD (Asseman *et al.*, 1999), suggesting that IL-10 has a role in either the development or action of these regulatory cells (Groux and Powrie, 1999). How regulatory cells are able to suppress other T-cells is poorly understood. Cytokines, especially TGF-β (Prudhomme and Piccirillo, 2000) and IL-10 (Powrie *et al.*, 1996; Shevach, 2000) are thought to be involved as is CTLA-4 (Powrie, 1999).

Regulatory T-cells are antigen specific and dependent; cells protecting from IBD will not develop in animals kept in clean conditions without a gut flora. Despite the involvement of IL-10 in the control of IBD, regulatory T-cells are distinct from Th2 cells, not least in their independence from IL-4 (Powrie *et al.*, 1996).

Recently a different distinct population of antigen specific double negative regulatory T-cells with the unique phenotype of $\alpha\beta TCR^+$, $CD4^-$, $CD8^-$, $CD25^+$, $CD28^-$, $CD30^+$, $CD44^-$ has been described (Zhang *et al.*, 2000). These cells are able to acquire alloantigen from APCs and present it to $CD8^+$ activated T-cells along with a death signal mediated by Fas via cell-to-cell contact (Zhang *et al.*, 2000).

Oral and nasal tolerance

The immune system continuously encounters many harmless but foreign environmental antigens. Most of these foreign but harmless antigens enter the body via the nose or mouth, and first encounter the immune system at a mucosal surface. Pollen, house dust mite (*Dermatophagoides pteronyssinus)* proteins, and food all contain potential antigens, but it is unnecessary and damaging to host tissue to mount an immune response to these antigens. Except in cases of allergy the immune system is tolerant of them. It is important to remember that not everything that enters via a mucosal route is harmless -. Many infectious diseases including HIV can be transmitted across mucosa (Miller *et al.*, 1989). Accordingly, the immune system is not tolerant of everything delivered to mucosa; the mucosal immune system can be activated by pathogens. In the case of *Chlamydia* infection, activation of the mucosal immune system contributes to pathogenesis (Rasmussen *et al.*, 1997; Stallmach *et al.*, 1998). However, an immune response to other mucosal pathogens including herpes simplex virus (Richards *et al.*, 1998), Mycobacterium (Falero-Diaz *et al.*, 2000), and in some cases HIV-1 (Mazzoli *et al.*, 1997; Kaul *et al.*, 1999; Clerici *et al.*, 1999) can be protective. Tolerance induced by antigens delivered to a mucosal surface is known as *mucosal (or oral or nasal) tolerance* (Wells and Osborne, 1911; Strobel and Mowat, 1998; Palliser *et al.*, 1998; Lowrey *et al.*, 1998). Just how the immune system 'knows' whether to ignore or react to a mucosally delivered antigen is poorly understood. The dose, length of exposure, presence or absence of microbial components and tissue damage (danger signals) must all play a role (Matzinger,

1994; Lowrey *et al.*, 1998; Chin *et al.*, 2000). Mucosal APCs are ideally placed to integrate these signals, migrate to draining lymphoid tissue, and cause T-cells to develop into activated Th1 or Th2 cells or anergic or regulatory cells by varying the signals which they give to antigen specific naive T-cells. For example, in the absence of infection, resident pulmonary alveolar macrophages inhibit T-cell activation (Thepen *et al.*, 1989) by the production of lymphostatic mediators (Holt, 1986), inefficient antigen processing and presentation (Holt, 1986), and an absence or reduction in co-stimulatory molecule expression (Chelen *et al.*, 1995). Other signals may also be involved.

Experimental intranasal delivery of peptides can induce transient activation of T-cells followed by the induction of clonal anergy (Tsitoura *et al.*, 1999), and the activation of regulatory T-cell populations (Palliser *et al.*, 1998).

Linked suppression, bystander suppression and APCs

Der p1 is an antigenic protein of the house-dust mite (*Dermatophagoides pteronyssinus*) and a common allergen in man and experimental animals. Antigenic epitopes of Der p1 have been identified in H-2^b mice using synthetic peptides representing short sequences of Der p1. Der $p1^{110-130}$ produces a peak antigenic response, and was therefore identified as containing the immunodominant epitope. Weaker responses were observed to Der $p1^{81-102}$, Der $p1^{21-49}$ and Der $p1^{197-212}$ (Hoyne *et al.*, 1994). Intranasal administration of Der p1 peptides is able to tolerise an animal to subsequent (2 weeks to 6 months) *in vitro* T-cell challenges with either the peptides or full length Der p1. The immunodominant epitope induced the most profound tolerance (Hoyne *et al.*, 1997). If animals were tolerised with the immunodominant peptide of Der p1 alone, their cells showed a reduced response to all epitopes of Der p1 on subsequent challenge with full length Der p1 (Hoyne *et al.*, 1997). This phenomenon, where induction of tolerance to one epitope induces non-responsiveness to other epitopes on the same molecule, is known as **linked suppression**. If mice in the Der p1 model were nasally tolerised with the immunodominant peptide of Der p1, and 14 days later co-immunised with Der p1 and OVA, their T-cells showed a reduced response to epitopes on both Der p1 and OVA (Hoyne *et al.*, 1997). This phenomenon, where induction of tolerance to one

antigen spreads to other antigens which are encountered by the immune system simultaneously to the tolerised antigen, is called **bystander suppression**.

If nasal tolerance induces regulatory / suppressor T-cells (there is some controversy over whether this is the case or if nasal tolerance is purely the result of T-cell anergy Whitacre *et al.*, 1991; Melamed and Friedman, 1993; Garside *et al.*, 1995), then a mechanism to explain linked and bystander suppression involving APCs becomes apparent. Recent studies (Yoshida *et al.*, 1997; Krause *et al.*, 2000) suggest that mucosal administration of high dose antigen results in clonal anergy, and that low dose antigen tolerises by inducing transient T-cell activation and subsequent induction of actively suppressing cells. When a regulatory T-cell (Tr-cell; figure 5.1) encounters its epitope on an APC, naive, potentially reactive, T-cells recognising the same or other epitopes simultaneously presented by the same APC will also engage their TCRs. Epitopes which are parts of the same protein or which are endocytosed by the APC at the same time are most likely to be co-presented. The close proximity of the T-cells on the same APC allows the regulatory T-cell to give a tolerising signal to naive T-cells engaged at the same APC. The nature of the tolerising signal that passes from the regulatory T-cell is unknown; figure 5.1 presents some possibilities.

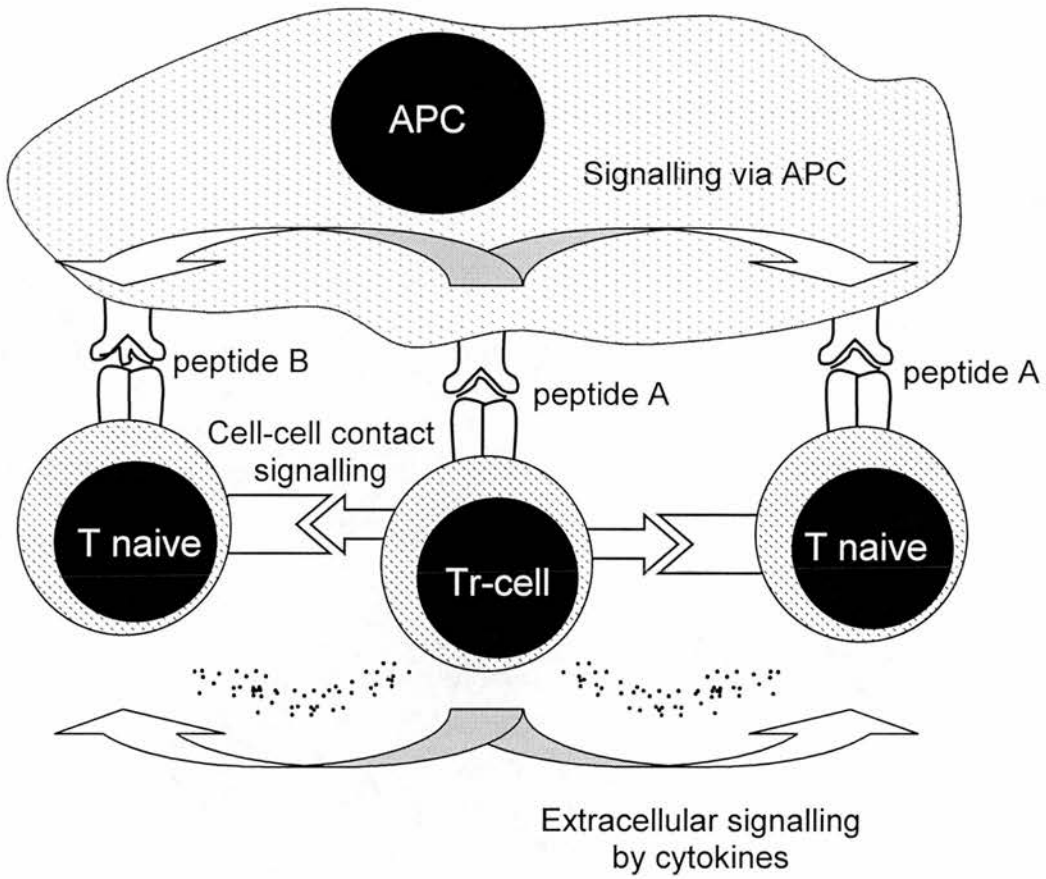


Figure 5.1. Models of linked and bystander suppression. A regulatory / suppressor T-cell (Tr-cell) recognises MHC / peptide A complex on an APC (for clarity CD4 and signal two are not shown). Naive T-cells recognising the same or a different epitope on the same APC can be tolerised and become anergic and/or Tr-cells themselves. There are three potential mechanisms by which the tolerance signal can be delivered to the naive T-cells. The signal could be propagated through the APC; the most obvious mechanism for this to occur would be if the APC down-regulated its co-stimulatory molecules on encountering a Tr-cell. Alternatively, naive T-cells could receive their peptide in a tolerance-inducing cytokine environment established by the Tr-cell (and/or the APC); it has been suggested that the Tr-cell could prevent the expansion of the naive T-cells by 'mopping up' IL-2 (Lombardi *et al.*, 1994; Lombardi *et al.*, 1995). In a third alternative mechanism, the proximity of the T-cells could allow a tolerogenic signal to be delivered by T-cell-to-T-cell contact. One, more or all of these mechanisms may operate *in vivo*.

Tolerance induction

The re-establishment of antigen-specific tolerance is the goal of the treatment of autoimmune disease. *In vitro* T-cells can be rendered non-responsive (anergic / regulatory) to antigen stimulation by treatment with very high doses of antigen, or when given antigen in the presence of non-depleting anti-CD4 or anti-CD8 (Qin *et al.*, 1993). *In vivo* oral administration of antigen can also result in clonal anergy. Experimental autoimmune encephalitis (EAE) is an animal model of multiple sclerosis (MS). The disease is induced in mice or rats by eliciting a Th1 $CD4^+$ T-cell response against myelin basic protein (MBP) by injection of MBP and adjuvant, which results in an MS-like phenotype (Kumar and Sercarz, 1998). Feeding MBP to affected animals causes oral tolerance and remission of disease (Metzler and Wraith, 1993) by inducing regulatory T-cells, which can be cloned and used to transfer protection to other animals. The regulatory T-cells are $CD4^+$ and have a similar specificity and epitope recognition to the encephalitogenic Th1-cells. They suppress the encephalitogenic cells by producing TGF-β and IL-10 (Chen *et al.*, 1994). Although they can produce varying amounts of IL-4, this cytokine does not appear important for protection because EAE can be ameliorated by oral administration of MBP in IL-4 knockout mice (Liblau *et al.*, 1997). In the Lewis Rat model of EAE, intra-tracheal administration of MBP induces tolerance with a higher potency than does oral administration (Pietropaolo *et al.*, 2000).

Tolerance and HIV-1

As reviewed in the introduction to this thesis, HIV-1 induces an immune deficit. Decline of leukocyte numbers, anergy, changes in APC cytokine production, a Th1 to Th2 switch and inappropriate apoptosis all contribute to the immunodeficiency seen in AIDS. Tolerance induction involves anergy, apoptosis and changes in cytokine production. It is therefore possible that some of the molecular signalling events involved in HIV-1 pathogenesis may be shared with those involved in tolerance induction. Indeed it could be claimed that HIV-1 has evolved to subvert the physiological mechanism of tolerance induction and immune regulation to its own ends in order to escape immune destruction.

Measurements of tolerance

A major hindrance to *in vitro* and *in vivo* tolerance experiments is that they attempt to measure the absence of a response, which is not always as easy to detect as a positive response (usually observed as a proliferation). Because tolerance is usually antigen specific, much work has been done with T-cell clones, T-cell lines and transgenic T-cells so that the absence of a response of a single specificity is not swamped by responses of other specificities. The HLA DR1*0101 restricted influenza haemagglutinin reactive T-cell clone HA1.7 (Eckels *et al.*, 1982; Lamb *et al.*, 1982a; Lamb *et al.*, 1982b) and the HLA DR 11 restricted Der P II $^{28\text{-}40}$ reactive T-cell clone AC1.1 (Pala *et al.*, 2000) were used in some of the tolerance experiments described here.

Notch and cell-to-cell interactions

The inner cell mass of a mammalian blastula is a clump of a few hundred cells. All of these cells are equivalent, genetically identical and totipotent (Walpert, 1991). The question at the heart of embryology is – "why do some of the descendants of these early cells differentiate into liver cells, some into skin cells, some into brain cells and some into blood cells?" When haematopoiesis is considered similar questions are raised, that is – "why do some blood cells become erythrocytes and others become lymphocytes?" Equivalent questions in the peripheral immune system are – "why do some initially equivalent T-cells become Th1 as opposed to Th2, or regulatory cells as opposed to effector cells, and why do some immune cells live rather than die?" An embryologist's answer to these questions is that pattern formation can be explained by induction reinforced by differentiation. Induction is the process where one region of the developing embryo interacts with a second region in order to influence the second region's differentiation, via cell autonomous and non-autonomous signalling events. Can this view be extended to our understanding of how cell fate decisions may be made in the peripheral immune system? If so, the immune system might be able to utilise many of the conserved

signalling pathways that are used during embryonic development, to regulate cell growth and fate decisions in mature lymphocytes.

In embryonic development inductive interactions can set up tissue patterning from initially equivalent cells due to slight and randomly occurring initial differences between cells. This is illustrated in figure 5.2. Figure 5.2a represents a tissue consisting of a single sheet of cells. All cells are equal and can send and receive a signal from their neighbours. Signal strength is governed by a simple rule – the more signal a cell receives the less signal it gives to its neighbours. In figure 5.2a all cells are giving and receiving an equal (medium) amount of signal to and from their neighbours. A random event perturbs this unstable equilibrium and one cell (starred) produces slightly more signal than its neighbours. This causes the surrounding cells to down-regulate their own signal (figure 5.2b). Two additional cells in figure 5.2b now receive less signal than the others and up-regulate their own signal in response (figure 5.2c). The pattern of signalling is propagated across the field (figure 5.2d), resulting in isolated signaller cells surrounded by receivers. This pattern can be made permanent by differentiation of signallers and receivers, thus giving rise to two distinct cell fates (figure 5.2e).

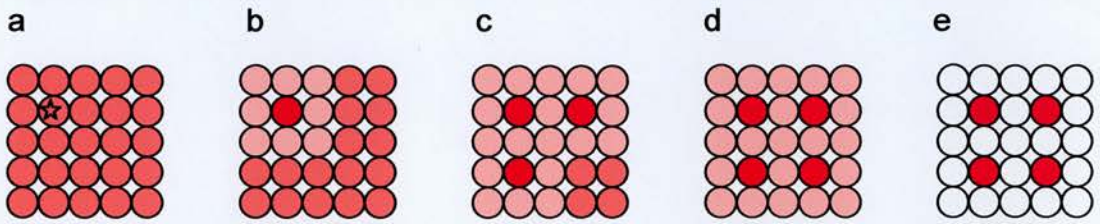


Figure 5.2. Establishment of tissue patterning in an equipotential field. The level of signal produced by each cell is indicated by the intensity of red shading.

Figure adapted from Gilbert, 1994. © Sinauer Associates Inc. 1994.

The example given in figure 5.2 was proposed to explain the development of the *Drosophila* ventral nerve cord (Greenwald and Rubin, 1992). Starting from a field of about 1800 equivalent ectodermal cells, one quarter will become neuroblasts and the rest hypodermis (Hartenstein and CamposOrtega, 1984). The initial differences

between the cell types is created by chance and amplified by induction; the cell types become arranged in a similar pattern to figure 5.2e (ArtavanisTsakonas *et al.*, 1991).

Inactivating mutations in two *Drosophila* genes, called *notch* and *delta*, result in embryo death because all the cells in the field become neuroblasts (Lehmann *et al.*, 1983; ArtavanisTsakonas *et al.*, 1991). Experiments with genetic mosaic embryos show that *notch* is needed in the cells that will become epidermis and *delta* is needed in the cells that will induce the epidermal phenotype. This is because Delta is a signalling molecule expressed on the cell-surface and Notch is its receptor. Could Notch and Delta be involved in signalling between immune cells? Such a suggestion is theoretically attractive if the immune system is considered as an organ that develops throughout the lifetime of the organism. Inputs to the system by chance antigen-encounters influence not just the cell which meets the antigen but the 'organ' as a whole and future patterns of immunity. Phenomena such as linked suppression, regulatory cell suppression, original antigenic sin and immune deviation can be viewed as a dynamic version of **pattern formation**.

Viewing the immune system as an integrated 'organ' or 'network' is not a new idea (Matzinger, 1994; Janeway and Travers, 1996). Traditionally communication between cells of the immune system has been viewed as cell-to-cell interaction mediated by exclusive 'immune system' signals and soluble cytokines. However, there is growing interest in the role of pattern formation genes in the establishment of immune system patterning.

The Notch signalling pathway

***Notch* genes.** The *notch* locus was first isolated from *Drosophila melanogaster* (Welshons, 1971) where heterozygous loss-of-function mutants have wing notches and homozygous loss-of-function mutants die due to the abnormality in neuronal induction described above (ArtavanisTsakonas *et al.*, 1995). One *Xenopus* homologue (*Xotch*) and four mammalian homologues (*notch-1* to *-4*) of *Drosophila notch* have been identified (Egan *et al.*, 1998). Other members of the Notch signalling pathway were identified in *Drosophila* by their genetic interaction with *notch* alleles and have since been found in mammals. Mutations in the *HES* (*hairy* and *enhancer of split*) genes cause enhancement of *notch* phenotypes

(CamposOrtega, 1991). Loss of the *deltex* gene suppresses the lethality of gain-of-function notch alleles such as *abruptex* (Busseau *et al.*, 1991; Busseau *et al.*, 1994). In *Caenorhabditis elegans* the *notch* homologue *lin-12* is involved in fate induction, lateral specification and cell division (Austin and Kimble, 1987; Yochem *et al.*, 1988; Yochem *et al.*, 1988). Human *notch-1* and *notch-4* were independently isolated as the *TAN-1* and *int-3* oncogenes (Ellisen *et al.*, 1991; Robbins *et al.*, 1992; Joutel and TournierLasserve, 1998).

Protein structure of mammalian Notches. Mammalian homologues of Notch are very large membrane-spanning glycoproteins. They have an N-terminal extracellular signal peptide and up to 36 EGF-like repeats (Wharton *et al.*, 1985; Kidd *et al.*, 1986; ArtavanisTsakonas *et al.*, 1995). Six of these repeats are predicted Ca^{2+} binding domains (Rao *et al.*, 1995) and in Notch-4 the EGF repeat region is rather different, suggesting a different ligand specificity (Uyttendaele *et al.*, 1996; Gallahan and Callahan, 1997; Egan *et al.*, 1998). The EGF repeats are followed by three copies of a cysteine rich Lin-12 / Notch repeat (LNR, Yochem *et al.*, 1988). Deletion of the LNRs results in constitutively activated Notch. It has been suggested that the LNRs prevent the dimerisation of Notch, required for activation, unless a Notch ligand is present (Kidd *et al.*, 1989; Lieber *et al.*, 1992; Lieber *et al.*, 1993; DeCelis and GarciaBellido, 1994; Egan *et al.*, 1998). The cytoplasmic domain of Notch contains a Ram domain, six ankyrin repeats and a PEST domain involved in protein turnover (Breeden and Nasmyth, 1987). There is also a cytoplasmic cleavage site, Notch being cleaved after activation (ArtavanisTsakonas *et al.*, 1995; Egan *et al.*, 1998).

Notch ligands. Several ligands for Notch have been identified (ArtavanisTsakonas *et al.*, 1995; Fleming *et al.*, 1997); the nomenclature varies across species (see Lissemore and Starmer, 1999, for a discussion of Notch ligand phylogeny). The names used here for all species are based on the names of the original *Drosophila* genes. *Serrate* (also called *jagged*) and *delta* (also called *delta-like, dll*) genes, of which there are five in mammals, encode proteins which act as Notch ligands (Nye and Kopan, 1995; Zimrin *et al.*, 1996). Notch ligands all share a cysteine-rich ~200 amino acid DSL (Delta / Serrate / Lag-2) conserved domain (Tax *et al.*, 1994). The DSL domain is important for Notch ligand function because it binds to EGF repeats 11 and 12 of Notch (DeCelis *et al.*, 1993). Other Notch, Delta and Serrate regions

may also influence binding events (Lieber *et al.*, 1992). Serrate and Delta are both membrane-anchored proteins with unrelated cytoplasmic tails (Egan *et al.*, 1998). They can signal to Notch whilst bound to the surface of the same or different cells, Serrate and Delta may possibly also be cleaved from their membranes and function as soluble signalling molecules (ArtavanisTsakonas *et al.*, 1995; Egan *et al.*, 1998).

Ligand specificity. Experiments with Notch and its ligands are complicated by the fact that the specificity of ligand for receptor is not well understood. Whether all of the Notches within a single species can bind all of the Serrates and Deltas, and whether this binding can initiate signalling in all cases is unknown. There is a confusing array of data in this regard with evidence coming from *in vitro* adhesion assays, mutation analysis and co-localisation of mRNA in tissues at the time of signalling. In *Drosophila* neuroblast differentiation (modelled in figure 5.2), Serrate can compensate for a loss of Delta (Gu *et al.*, 1995). However, in wing development, Delta and Serrate have distinct and non-overlapping capabilities (Doherty *et al.*, 1996). In mammals there is evidence for distinct but overlapping ligand pairs. In the developing rat hindbrain and spinal cord, *serrate-1* and *delta-1* are expressed in well-demarcated stripes (Lindsell *et al.*, 1996). Co-localisation of mRNA with *notch* genes suggests that *delta-1* interacts with *notch-1* and *serrate-1* with *notch-3* in order to specify different cell fates (Lindsell *et al.*, 1996). In binding studies with mouse proteins, Serrate-1 can bind to Notch-1, -2 and -3 (Shimizu *et al.*, 1999). In a mouse myoblast system (Weinmaster, 1998), Delta-1 activates Notch-1 but not Notch-2, and Serrate-1 activates both Notch-1 and -2. A cysteine-rich region – unique to Serrate-1 – appeared to be required for Notch-2 signalling. If this region is removed from Serrate-1, Notch-2, but not Notch-1, signalling is prevented. Conversely, adding a cysteine rich region to Delta-1 allows it to signal through both Notch-1 and Notch-2 (Weinmaster, 1998). Receptor-ligand pairing in other systems may be completely different.

Notch activation

Dominant mutant alleles of various *notches* have been identified in which all or most of the sequence encoding the extracellular domain is missing (Ellisen *et al.*, 1991; Robbins *et al.*, 1992; Rohn *et al.*, 1996). Cell-lines have also been transfected with

constructs based on the intracellular domain of Notch-1 (Callahan *et al.*, 2000). In all these cases the intracellular portion of the Notch molecule, in the absence of the extracellular domain, is constitutively active (Egan *et al.*, 1998). One result of this activation is (usually) the prevention of apoptosis, which may explain why alleles of *notch* with these deletions have been isolated from tumours (Ellisen *et al.*, 1991; Robbins *et al.*, 1992). EBV nuclear antigen 2 appears to transform B-cells by interacting with the CBF-1 / RBPJκ transcriptional repressor in a similar way to Notch (Hsieh *et al.*, 1996; Strobl *et al.*, 1997; Callahan *et al.*, 2000).

When Notch binds to its DSL containing ligands there is proteolytic cleavage of the Notch protein (figure 5.3) which results in the release of the active intracellular region. The cleavage protease has not yet been identified with certainty; Furin or the metalloprotease Kuzbanian may be responsible (Pan and Rubin, 1997; Weinmaster, 1998). It is likely that the protease is constitutively present and active but that ligand binding is required to disrupt Notch dimers and expose the cleavage site. In an alternative model of Notch activation (Blaumueller *et al.*, 1997) newly synthesised Notch is cleaved in the *trans*-Golgi before reaching the surface. Following cleavage, the extracellular and transmembrane-intracellular fragments remain non-covalently associated and travel to the plasmalemma. DSL-ligand binding causes breakdown of this association and the release of the extracellular domain (a second cleavage may mediate this). The intracellular domain, (possibly by a second (or third) cleavage (Schweisguth, 2000)), is then released from membrane anchorage and becomes active. In this model ready-cleaved Notch is on the cell surface, therefore it is possible that under certain physiological circumstances (reducing conditions) the non-covalent association between the two Notch peptides could be disrupted, releasing soluble Notch fragments which could neutralise Notch ligands (Blaumueller *et al.*, 1997). According to either model, following signalling the extracellular domain of Notch may remain bound to the DSL-ligand expressing cell or be endocytosed by this cell. This trans-endocytosis can cause confusing experimental results with Notch ligands being identified by immunocytochemistry in cells that have not synthesised it.

Activated Notch targets – HES-1

Activated Notch causes the transcription of nuclear genes including the *HES* (*hairy* and *enhancer of split*) genes in *Drosophila* and vertebrates (DeCelis *et al.*, 1996; Jarriault *et al.*, 1998) by releasing the transcription factor CBF-1 from its association with membrane anchored Notch allowing it to bind the TGGGAA promoter nuclear sequence (Knust *et al.*, 1987; Tamura *et al.*, 1995; Lu and Lux, 1996). It is not known whether activated CBF-1 is associated with an intracellular cleavage fragment of Notch when it enters the nucleus. The *HES* genes encode basic helix-loop-helix transcription factors (Delidakis *et al.*, 1991). HES proteins complex with the widely expressed Groucho repressor (Paroush *et al.*, 1994) and can repress genes with CACNAG promoter sequences (Tietze *et al.*, 1992). Thus, this branch of Notch signalling can result in both transcriptional activation and repression. Targets for activation by HES / CBF-1 include *Notch* (Weinmaster, 1998) and *NF-κB* (Oswald *et al.*, 1998), a transcription factor involved in many immune processes including the increased expression of inflammatory cytokines, co-stimulatory molecules, and molecules involved in antigen processing and presentation. Specific targets of NF-κB include IL-1, -2, -3, -8, -12, TNF-α, IFN-β, GM-CSF, M-CSF, G-CSF, TAP1, MHC class I and II, and β_2-microglobulin (May and Ghosh, 1998). NF-κB is also involved in inducing tolerance to LPS in B-cells (Wedel *et al.*, 1999), monocytes (Frankenberger and ZieglerHeitbrock, 1997) and endothelium (Lush *et al.*, 2000). In B-cells at least, NF-κB can cause the up-regulation of *serrate* (Bash *et al.*, 1999). In the context of HIV-1 infection, NF-κB is both up-regulated in T-cells by interaction with gp120 (Briant *et al.*, 1998) and acts on HIV-1 provirus to increase viral gene transcription (Alcami *et al.*, 1995). There is also evidence that the NF-κB2 isoform of NF-κB (Oswald *et al.*, 1998) and GM-CSF (Cockerill *et al.*, 1996) can be expressed by the action of CBF-1 in the absence of HES. NF-κB2 activity has been associated with protection from apoptosis in lymphoid tissues (Osborne and Miele, 1999).

Interestingly HES also binds to a silencer site in the *CD4* promoter and acts to reduce *CD4* gene expression in T-cells (Kim and Siu, 1998).

Activated Notch targets – Deltex

Deltex is a cytosolic protein, present in both *Drosophila* and vertebrates, which is able to interact with Notch via the ankyrin repeats of Notch's intracellular domain (Diederich *et al.*, 1994). Deltex expression acts as a positive regulator of Notch signalling through CBF-1 and HES, possibly by disrupting Notch / CBF-1 interactions and thereby inhibiting the cytoplasmic retention of CBF-1 and encouraging its translocation to the nucleus (Matsuno *et al.*, 1995).

When *CBF-1* and *HES* are mutated in *Drosophila*, some but not all of the actions of Notch are inhibited (DeCelis *et al.*, 1996; Matsuno *et al.*, 1997). These data suggest that not all of the targets of Notch signalling are influenced via CBF-1-mediated signalling. It has been suggested (Matsuno *et al.*, 1995; Matsuno *et al.*, 1997) that Deltex has a second role in transducing Notch signalling into CBF-1-independent downstream events via its association with Grb-2. Grb-2 is a molecular adaptor protein that links receptor tyrosine kinases to *ras* signalling and ultimately nuclear transcription events (Lowenstein *et al.*, 1992). Grb-2 is involved in several immunologically important signalling processes especially in monocytes and macrophages. Grb-2 (and in some cases a related protein, Mona, Bourette *et al.*, 1998) is involved in transducing signals from M-CSF and GM-CSF receptors to ras and other growth and differentiation controlling genes by binding to phosphorylated signalling molecules such as STAT-5 (Odai *et al.*, 1997; Rohrschneider *et al.*, 1997; Yeung *et al.*, 1998; Yagisawa *et al.*, 1999). Grb-2 also binds to MAP kinases thereby mediating macrophage responses to the anaphylatoxin C5a (Torres and Forman, 1999). Fcγ receptor signalling, leading to macrophage activation and the production of reactive oxygen species is dependent on the adaptor function of Grb-2 (ErdreichEpstein *et al.*, 1999). Monocyte IL-3 responses (Anderson *et al.*, 1997; Yagisawa *et al.*, 1999), eosinophil IL-5 responses (Bates *et al.*, 2000) and the activation of T-cells by CD43 cross-linking (PedrazaAlva *et al.*, 1998) all involve Grb-2 in their signal transduction pathways. Deltex may also be involved in suppression of gene transcription. A proposed mechanism of Deltex action is that Deltex / Grb-2 heterodimers inhibit JNK-mediated activation of the transcription factor E47 (Ordentlich *et al.*, 1998). E47 is a basic helix-loop-helix protein involved

in B-cell specific Ig gene transcription and is required for early B-cell development (Zhuang *et al.*, 1994; Bain *et al.*, 1994).

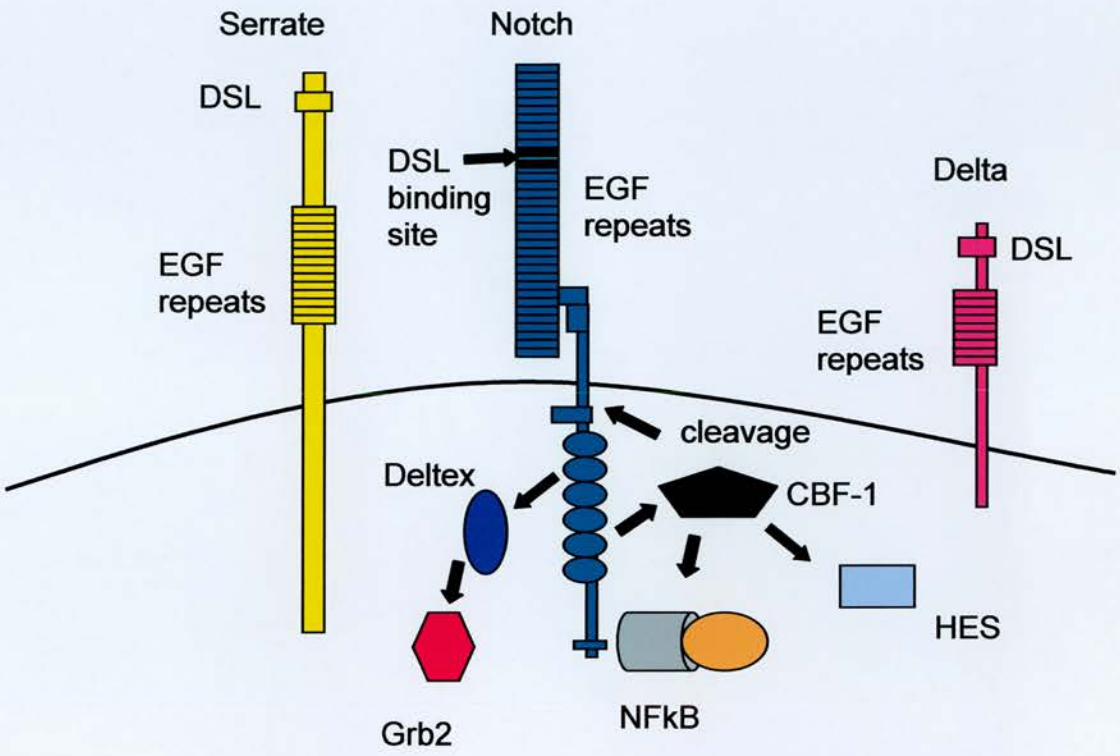


Figure 5.3. The Notch signalling pathway. Notch ligands Delta and Serrate (usually on a different cell, rather than the same cell as Notch as shown here) bearing the DSL domain bind to EGF repeats of the extracellular domain of Notch. This causes cleavage of Notch, possibly resulting in trans-endocytosis of the extracellular domain, and nuclear translocation of the intracellular domain to the nucleus. The intracellular domain is involved in inducing the transcription of genes such as HES, Deltex and NF-κB.

Integration with other pathways

The Notch pathway interacts with a variety of other signalling pathways. In addition to the potential to modulate NF-κB, MAP kinase and STAT signals as discussed above, the Notch pathway interacts with other proteins which were also first

described as developmentally important in *Drosophila*, but have since been identified in mammals.

Fringe proteins (**lunatic**, **radical** and **manic**) and the neurogenic protein **Brainiac** are not DSL-motif containing -Notch ligands, but secreted proteins able to interact with the extracellular domains of Notch ligands, possibly by changes to gylcosylation so as to abrogate Serrate, but potentiate Delta signalling (Fleming *et al.*, 1997; Wilson *et al.*, 1997; Panin *et al.*, 1997; Egan *et al.*, 1998; Wu and Rao, 1999). Recent studies suggest that fringe is a glycosyltransferase (Brückner *et al.*, 2000) which exhibits most of its Delta modifying activity in the Golgi apparatus before it is secreted (Munro and Freeman, 2000). During vertebrate embryogenesis bursts of **Lunatic Fringe** expression cause periodic activation of Notch and regulate body segmentation, the so called 'segmentation clock' (Pourquié, 1999). The intracellular adaptor protein **Numb** can block Notch signalling (CamposOrtega, 1996; Spana and Doe, 1996; Guo *et al.*, 1996) presumably by binding to its intracellular domain, although studies in cultured cells have failed to demonstrate Numb blockade of CBF-1 binding (Frise *et al.*, 1996; CamposOrtega, 1996). **Dishevelled** is a protein required for the reception of the **Wingless** signal and is phosphorylated in response to this signal (Yanagawa *et al.*, 1995). Dishevelled can also bind to the intracellular domain of Notch, and inhibit Delta signalling through Notch (Axelrod *et al.*, 1996). **Hairless** interferes with Notch signalling by blocking the association of CBF-1 with its promoter target sequences (Bang *et al.*, 1995); the phosphorylation state of Hairless may regulate this activity (Christensen *et al.*, 1996). **Bearded** may also regulate Notch signalling by modulating Notch / CBF-1 interactions (Leviten and Posakony, 1996). Bone morphogenetic proteins (**BMPs**) can cause a reduction in *delta* transcription by interfering with achaete / scute transcription factors involved in the regulation of *delta* expression (Wilson and HemmatiBrivanlou, 1997). Proteins such as **Noggin** and **Chordin** can interfere with BMP function and therefore may be capable of increasing Delta protein expression (Lamb *et al.*, 2000).

The rapid expansion of molecules known to interact with Notch signalling will undoubtedly continue over the coming years as more proteins are identified by

genetic screens and *in vitro* binding assays, and assisted by the Human Genome Project, their mammalian homologues are identified.

Notch in immune system development

Most work on Notch signalling has focused on its roles in embryogenesis where it is particularly important in neurogenesis (Xu *et al.*, 1990; ArtavanisTsakonas and Simpson, 1991; ArtavanisTsakonas *et al.*, 1991). Other roles beyond neurogenesis and wing development include the role of the Notch pathway in immune system development. Notch and its ligands are widely expressed in immune cells and tissues. $CD34^+$ haematopoietic precursors express Notch-1 and -2 (VarnumFinney *et al.*, 1998). Double negative thymocytes express high levels of Notch-1, double positives express little or no Notch-1, and mature $CD4^+$ and $CD8^+$ T-cells express intermediate levels of Notch proteins (Hasserjian *et al.*, 1996; Felli *et al.*, 1999). Thymus and bone marrow stromal cells express some (but not all) Notch ligands (Li *et al.*, 1998; VarnumFinney *et al.*, 1998; Felli *et al.*, 1999), and isolated cells in the periarteriolar sheath but not the germinal centres of mouse spleens express Delta-1, Serrate-1 and Notch-1 (Lamb *et al.*, 1998). mRNA for Notch-1 and Serrate-1 has been detected in splenic $CD4^+$ and $CD8^+$ T-cells, B-cells and DCs (Hoyne *et al.*, 2000). Taken together these data suggest that the potential for regulation of leukocyte development by Notch signalling exists. The differentiation stage of the cell receiving Notch signals appears to be highly important to the outcome of signalling, and would explain how the same pathway is able to influence many different cell fate decisions.

Notch and early haematopoiesis. When $CD34^+$ haematopoietic precursors were transfected to express constitutively activated Notch-1, or exposed to a Serrate-2 expressing cell-line, acquisition of granulocytic and myeloid differentiation markers was delayed (Carlesso *et al.*, 1999). Exposure to Serrate-1 expressing cells led to a proliferation of undifferentiated precursors (VarnumFinney *et al.*, 1998). These data suggest that in very early haematopoiesis (the $CD34^+$ precursor stage) the role of Notch signalling is to maintain and amplify undifferentiated precursor cells.

If *notch-1* is inactivated using the *Cre*-Lox system, bone marrow derived lymphocyte precursors are unable to develop into T-cells. B-cell development is

normal suggesting an instructive role for Notch-1 activation in inducing a T-cell rather than a B-cell fate (Radtke *et al.*, 1999). This observation is in line with constitutively active alleles of Notch-1 being responsible for human T lymphoblastic leukaemia (Ellisen *et al.*, 1991) and bone marrow precursors transfected with constitutively active Notch-1 producing extra T-cell precursors at the expense of B-cell precursors (Pui *et al.*, 1999).

Notch and αβ versus γδ T-cell development. Transgenes encoding constitutively active intracellular fragments of Notch can be transfected into developing thymocytes where they support differentiation to αβ T-cells at the expense of γδ T-cells (Washburn *et al.*, 1997). Expression of Notch ligands on thymic stromal cells may promote αβ T-cell production (Hayday *et al.*, 1999). In mice reconstituted with a 1:1 mixture of heterozygous Notch-1 +/- and Notch-1 +/+ haematopoietic precursors more of the γδ T-cells develop from Notch-1 +/- precursors than from the Notch-1 +/+ population (Robey and Fowlkes, 1998). αβ or γδ lineage commitment requires the production of a functional (rearranged in-frame and capable of signalling) TCR; Notch activation does not abrogate this requirement (Hayday *et al.*, 1999). It may be that Notch signalling is not instructive but acts by selectively enhancing the survival of αβ cells normally destined to die. Alternatively it may be a 'facilitative factor' modulating the cell's capacity to respond to instructive signals.

Notch and CD4 versus CD8 T-cell development. There has been long-running speculation as to how double positive thymocytes 'choose' CD4 or CD8 expression and how the choice of coreceptor matches the TCR specificity for MHC class I or II. Instructive and stochastic models of CD4 versus CD8 lineage decision have been proposed (Davis *et al.*, 1993; Chan *et al.*, 1993a; Chan *et al.*, 1993b; Suzuki *et al.*, 1995). Recently a role for Notch has been suggested (Deftos *et al.*, 1998), although the absence of Notch in double positive thymocytes (Hasserjian *et al.*, 1996) casts doubt on the physiological significance of this. Constitutively activated Notch-1 in mouse thymocytes leads to the production of extra $CD8^+$ T-cells and fewer $CD4^+$ T-cells (Robey *et al.*, 1996; Robey, 1999). BrdU labelling experiments in which non-proliferating double positive thymocytes are followed as they develop into single positives show that the extra $CD8^+$ cells are not simply the result of inappropriate single positive proliferation, but that a proportion of T-cells which normally chose

the CD4 lineage become $CD8^+$ T-cells (Robey, 1999). It appears that inappropriate Notch-1 signalling causes some MHC class II recognising T-cells, which would normally develop as $CD4^+$ T-cells to develop as (presumably non-functional) $CD8^+$ T-cells (Robey *et al.*, 1996). Robey, 1999, proposes a model of instructive T-cell subset commitment based on Notch expression linking coreceptor engagement to differentiation. Robey suggests that Class I recognition (CD8 engagement) by a double positive thymocyte leads to enhanced Notch signalling by DSL-containing Notch ligands on stromal cells and that this reinforces CD8 and suppresses CD4 expression (Robey *et al.*, 1998). Pertussis toxin is able to induce an increase in CD8 lineage selection by an up-regulation of Notch expression (Takahama *et al.*, 1997). There is also evidence that the Notch pathway intermediate HES-1 silences *CD4* gene expression in $CD4^+$ Th-cells (Kim and Siu, 1998).

Notch and apoptosis. Notch engagement can function as an anti-apoptotic signal (Miele and Osborne, 1999; Yang and Ashwell, 1999). Evidence for this comes from the ectopic expression of constitutively active Notch in some neoplasms (Ellisen *et al.*, 1991; Smith *et al.*, 1995; Gallahan and Callahan, 1997). *Notch-1* knockout mice die before birth, but inducible knockouts have been made using the *Cre*-Lox system. Following *Notch-1* gene excision mice exhibit overall size reduction, a smaller thymus and fewer single and double positive thymocytes (Swiatek *et al.*, 1994; Radtke *et al.*, 1999). Experimental expression of active Notch-1 in primary thymocytes and thymoma cell-lines results in increased expression of the anti-apoptotic gene *Bcl-2* and resistance to glucocorticoid induced 'death by neglect'. However, the protection from apoptosis offered by active Notch-1 cannot be so straightforward as the up-regulation of *Bcl-2* because induced expression of this gene alone does not prevent death by neglect (Deftos *et al.*, 1998). Active Notch-1 expression can also render T-cell lines refractory to TCR-mediated apoptosis by binding to the pro-apoptotic nuclear hormone, Nur77 and inhibiting its action (Jehn *et al.*, 1999). It is conceivable that Notch-mediated inhibition of apoptosis in the peripheral immune system could contribute to the specification of memory cell fate.

Little work has been done to study the influence of Notch on apoptosis of monocytic cells, and the picture here is complicated by the influence of cytokines. When primary human monocytes expressing high amounts of Notch-1 and -2 were

triggered *in vitro* with immobilised Delta-1 apoptosis resulted, but only in the presence of M-CSF and not GM-CSF (Ohishi *et al.*, 2000). As discussed above, Notch signalling can induce these cytokines and the G-CSF signalling pathway can interact with the Notch pathway via Grb-2. G-CSF, GM-CSF and Notch interact in myeloid differentiation. In 32D myeloid progenitor cells active Notch-1 specifically inhibits granulocytic differentiation induced by G-CSF, and Notch-2 inhibits GM-CSF-mediated differentiation (Bigas *et al.*, 1998). Specificity of the Notch effects is controlled by the Notch cytokine response (NCR) region of the cytoplasmic Notch protein (Bigas *et al.*, 1998). These results are hard to reconcile with another study where Notch-1 activation (by transfection with constitutively active *notch-1*, or co-culture with Serrate-1 expressing fibroblasts) accelerated granulocytic differentiation of 32D cells (Schroeder and Just, 2000). There is a danger that ectopic Notch expression experiments may produce artefactual results by inducing expression of Notch at non-physiologically high levels. Notch-1 knockout mice show normal monocyte, granulocyte, NK-cell and B-cell development, and thymic DCs develop normally despite a deficiency in the thymocyte lineage (Radtke *et al.*, 2000).

Notch and proliferation. As discussed above and in a similar manner to Notch / Delta interactions maintaining neuronal precursors in *Drosophila* (Brennan *et al.*, 1999), Notch / Serrate interactions can contribute to the maintenance of haematopoietic precursors (Carlesso *et al.*, 1999). However, under different circumstances, such as the absence of growth factors, Notch activation of human $CD34^+$ cells by cells expressing Serrate-1 inhibits proliferation (Walker *et al.*, 1999).

The outcome of Notch signalling is highly dependent on the other signals present – in many circumstances Notch signalling may simply acts as a 'facilitation factor'.

> *"The receptor encoded by the Notch gene plays a central role in preventing cells from making decisions about their fates until appropriate signals are present. ... Loss of Notch... function results in cells making premature and incorrect cell fate decisions, whilst increases in Notch signalling prevent cells from making these decisions."*
>
> Brennan *et al.*, 1999.

Notch and tolerance

About five years ago Lamb, Hoyne and Dallman proposed that Notch signalling might help to explain the induction of immunologic tolerance (Hoyne *et al.*, 2000). Invoking the Notch pathway to explain tolerance is theoretically appealing because all the components of the pathway appear to be expressed in the relevant cells and to be involved in haematopoiesis and immune system development (see above). Notch regulated fate specification and lateral inhibition in the embryo can be likened to processes involved in the induction of tolerance. Immune-dominance, bystander and linked suppression are all processes in which lymphocytes regulate the activity of other lymphocytes, either directly or via an APC. Cell proximity at an APC would allow cell to cell contact signalling, possibly by Notch. The experimental evidence accumulating in support of Notch's role in tolerance is summarised below (**N.B.** not all of this data has been published in peer-reviewed journals, sources include personal communications, abstracts, seminars at the University of Edinburgh and the Scottish Immunology Group 2000 meeting in St. Andrews and UK patent application GB2335194A).

- Mice were immunised with Der $P1^{110-131}$ peptide (the immunodominant epitope) or OVA. A week later the antigen-primed lymph node cells (LNC) were removed. *In vitro* proliferation and IL-2 production by the LNC in response to either recall antigen was reduced by more than 80% if a Der $P\ 1^{110-131}$ specific T-cell hybridoma, transfected to express surface Delta-1 and irradiated to prevent proliferation or cytokine secretion, was also present in the culture. Control hybridomas not expressing Delta-1 did not suppress LNC responses (Lamb *et al.*, 1998). In this experiment the Delta-1 expressing hybridoma did not inhibit T-cell responses specifically; this contradicts other data (see below) and models of suppression, and may have been due to the artificially close proximity of the suppressor and suppressed cells in culture.
- Murine splenic DCs were transfected to express Serrate-1 and pulsed with Der $P1^{110-131}$ peptide. Mice were then immunised with these DCs and boosted with Der P1 two weeks later. Seven days after boosting LNCs were isolated. LNCs from mice immunised with the Serrate-1 expressing DCs failed to proliferate or

secrete IL-2 when challenged *in vitro* with their recall antigen compared to cells from mice immunised with control DCs (Lamb *et al.*, 1998; Hoyne *et al.*, 2000). Potentially reactive T-cells had been tolerised (or deleted) when presented peptide by Serrate-1 expressing APCs. Further studies (Hoyne *et al.*, 2000) have shown that this tolerance lasts for at least 12 weeks, the longest time-point tested.

- Mice were injected with a Der $P1^{110-131}$ reactive, irradiated T-cell hybridoma, transfected to express Delta-1, at the same time as whole Der P1 protein in CFA. Seven days later LNCs were isolated and stimulated *in vitro* with Der $P1^{110-131}$ or Der P 1^{81-102} peptide. No IL-2 or proliferative response was seen in response to either epitope in LNCs isolated from mice treated with the Delta-1 expressing hybridoma, LNCs from mice treated with control transfected hybridomas did not show reduced antigenic responses (Lamb *et al.*, 1998). This experiment demonstrates Delta-1's role in eliciting linked suppression; although the Delta-1 expressing T-cell was specific for an epitope in Der $P1^{110-131}$ it was able to induce tolerance to another, linked epitope. It appears that tolerance induction towards the Der $P1^{81-102}$ epitope is a true example of linked suppression and not simply due to a non-specific inhibitory effect of the Delta-1 transfected hybridoma. A separate experiment (Lamb *et al.*, 1998) demonstrated that the Der P1-specific *delta-1* transfected hybridoma was unable to inhibit an *in vitro* recall response to the unrelated OVA protein (Lamb *et al.*, 1998; Hoyne *et al.*, 2000).
- The HA1.7 human T-cell clone (reactive to influenza haemagglutinin (HA) 306-318) was transfected to express cell surface Delta-1. These cells were mixed with normal HA1.7 cells, presentation competent APCs (irradiated HLA DRB1*0101 PMBCs) and $HA^{306-318}$ peptide, and were able to inhibit responses to antigenic stimulus in the non-transfected T-cells (Lamb *et al.*, 1998). The untreated HA1.7 cells retained a normal ability to proliferate in response to IL-2 (Lamb *et al.*, 1998). This experiment suggests that the Delta-1 expressing HA1.7 cells acted as suppressor cells and inhibited the response of other HA1.7 cells by Delta-1 mediated signalling. Presumably the suppressive signal was delivered by cell-cell contact when T-cells were brought into close proximity by interaction with APCs.

- HA1.7 cells proliferate in response to $HA^{306-318}$ peptide presented on L-cells (a fibroblast cell line transfected to express HLA-DRB1*0101). When the L-cells were transfected to express Serrate-1 in addition to HLA-DRB1*0101 the HA1.7 proliferative response was poor. The HA1.7 T-cells that had been tolerised by Serrate-1-expressing L-cells became suppressor T-cells themselves and were able to inhibit proliferation of fresh HA1.7 cells in response to antigen presented by normal APCs (irradiated PBMC, Lamb *et al.*, 1998). HA1.7 cells could act as suppressors after irradiation, which makes cytokine involvement unlikely and suggests involvement of signalling by cell-cell contact (Lamb *et al.*, 1998).
- When HA1.7 cells are cultured in the presence of a high dose of $HA^{306-318}$ peptide in the absence of APCs, a state of unresponsiveness to subsequent antigenic challenges is induced (i.e., tolerance). RT-PCR shows that resting HA1.7 cells do not express any transcript for Delta-1, but that 2 hours from the initiation of the tolerisation protocol *delta-1* mRNA appeared (Lamb *et al.*, 1998). No equivalent data has been reported to show an increase in Notch ligand expression following tolerisation by a Delta or Serrate expressing APC.
- Antigen-specific tolerance can be induced in mice by intranasal administration of the immunodominant Der $P1^{110-131}$ peptide (Hoyne *et al.*, 1993). Mice treated in this way or with PBS as a control and subsequently rechallenged subcutaneously with full length Der P1 in CFA were killed at various times following rechallenge and their superficial lymph nodes and spleens harvested for immunohistochemistry. Mice that received intranasal Der $P1^{110-131}$ peptide showed increased spleen and lymph node expression of Notch-1, Delta-1 and Serrate-1 from eight days after rechallenge (Lamb *et al.*, 1998). Control, PBS treated mice showed lower levels of these proteins (Lamb *et al.*, 1998).
- Mice previously immunised with Der P1 mount a strong *in vitro* recall response. However, a 75% reduction in this recall response was observed if the animals were immunised as normal and then tolerised with Serrate-1^{+} Der p $1^{110-131}$ pulsed DCs (Hoyne *et al.*, 2000). This experiment shows that Notch signalling is able to inhibit established immunity, an important demonstration if attempts are to be made to modulate the Notch pathway as a therapy for allergy or autoimmunity.

One criticism of the above experiments is that transfected cells may express Notch ligands at unusually high levels, which may not be relevant to physiologically induced tolerance. It would be interesting to see if physiological stimuli (e.g., oral tolerance, prostaglandins etc) or disease processes (e.g., the HIV-1 induced immune deficit) were able to induce changes in Notch ligand expression sufficient to see the tolerisation effects described above.

There is considerable commercial interest in modulating Notch signalling to therapeutic ends (Montesano *et al.*, 1997; ArtavanisTsakonas and Matsuno, 1997; Lendahl *et al.*, 1999; ArtavanisTsakonas *et al.*, 2000; Anon., 2000; Lamb *et al.*, 2000). Notch signalling could be modulated to induce angiogenesis following ischemia or wounding or to inhibit it in tumours (Uyttendaele and Kitajewski, 1998), as well as modulating the immune response to pathogens, allergens, xenografts, allografts or self-tissue in autoimmunity (Lamb *et al.*, 1998).

Semen, prostaglandin and tolerance

An immune response to spermatozoa is a threat to fertility (Kelly, 1999). Sperm express MHC class I antigens on their surface (Martinvilla *et al.*, 1996), although this has not been shown definitively (Vince and Johnson, 1995; Kelly, 1997). The presence of infection in either the male or female genital tract might provide a 'danger signal' (Matzinger, 1994; Medzhitov and Janeway, 1997) to the immune system and sensitise the female to subsequent sperm exposure in the absence of infection (Kelly, 1997). The evolutionary strategy adopted by man and other primates to prevent anti-sperm immune responses is to suppress the immune system of the female genital tract at the time of insemination (Kelly, 1997). This brings the risk that the female may not be able to mount an effective response to invading pathogens, especially sexually transmitted diseases. This risk may be minimised by secretory IgA and innate immune mechanisms such as macrophage phagocytosis in the male and female reproductive tracts and, the innate activity of anti-microbial chemicals in semen (Kelly, 1999).

It has long been recognised that human semen is profoundly immunosuppressive (see James and Hargreave, 1984; Alexander and Anderson,

1987, and Kelly, 1995, for reviews). It has also been suggested that semen is 'tolerogenic' and that repeated exposure to sperm antigens in the presence of seminal plasma will induce antigen-specific anergy and regulatory T-cell populations (Kelly, 1997; Kelly, 1999). Support for this comes from an epidemiological study of the incidence of preeclampsia, a condition associated with dysfunctional embryo implantation. The condition is significantly less common in women who have cohabited with the father of their child for at least 12 months before becoming pregnant (Robillard *et al.*, 1994). A possible reason for this is that repeated exposure to their partner's semen before conception had induced tolerance of antigens present on their partner's sperm and paternally encoded antigens on the embryo. Immune mediators in semen include spermine, prostaglandins and TGF-β (Kelly, 1999). The concentration of prostaglandin in human semen is 10,000 fold higher than that found in any other tissue and represents a considerable evolutionary investment by the male (Kelly, 1999). Low levels of IL-8, IL-6 and other cytokines are sometimes found in seminal plasma, but are produced by leukocytes in cases of leukospermia, rather than being intrinsic components of the ejaculate (Shimoya *et al.*, 1993; Paradisi *et al.*, 1997).

Prostaglandins (PGs) are usually viewed as pro-inflammatory mediators. Non-steroidal anti-inflammatory drugs such as indomethacin can inhibit their production and the resultant inflammation (Kelly, 1999). The pro-inflammatory action of PG is due to its vasoactive properties (Mossmann, 1973). The E series of PG (PGE) also have direct immunosuppressive actions on leukocytes by increasing intracellular cAMP levels so as to raise the threshold for activation (Kelly, 1999). PGE suppresses human T-cell proliferation (Goodwin *et al.*, 1977) by reducing IL-2 production (Chouaib and Fradelizi, 1982) and the expression of IL-2 receptor (Krouse and Deutsch, 1991). PGE is also immunosuppressive by other effects on cytokine release. On whole human blood IL-12 release is inhibited (Kraan *et al.*, 1995) and IL-10 production enhanced (Kelly *et al.*, 1997a) by PGE and 19-hydroxy-PGE (19HO-PGE), both major components of semen (Kelly, 1997). Both IL-10 (Chang *et al.*, 1995; Iglesias *et al.*, 1997) and PGE (Iglesias *et al.*, 1997) down regulate B7 expression on APCs, an addition possible mechanism of tolerance induction. PGE has an established role in inducing tolerance of gut microflora.

Transgenic mice engineered to contain only hen egg lysozyme reactive $CD4^+$ T-cells are tolerant of the orally administered protein. However tolerance cannot be established, and gut pathology is seen, if indomethacin is concurrently administed to the mice (Newburry *et al.*, 1999; Morteau, 1999; Murch, 2000). Indomethacin is an inhibitor of cyclooxygenase-2 (COX-2), an enzyme involved in PG production (Newburry *et al.*, 1999). In humans with IBD COX inhibitors have been shown to exacerbate intestinal inflammation (Kauffman and Taubin, 1987; Aabakken and Osnes, 1989; Bjarnason *et al.*, 1993).

HIV-1, gp120 and prostaglandin

Many viruses are known to manipulate the immune system towards tolerance (Alcami and Koszinowski, 2000). EBV produces an IL-10 mimic (Viera *et al.*, 1991). Measles virus inhibits IL-12 production by infected monocytes (Karp *et al.*, 1996), and CMV stimulates PGE production by host cells (Nokta *et al.*, 1996). Male to female sexually transmitted HIV-1 may avoid the need to manipulate the immune system in this way by being delivered in the tolerogenic environment of semen (Kelly, 1997). Jay Levy has long studied a soluble factor produced by $CD8^+$ T-cells which blocks HIV-1 infection of cells (Levy *et al.*, 1996). Although the identity of this factor has yet to be identified, its production is blocked by IL-10 (Barker *et al.*, 1995). PGE in semen has the potential to abolish production of this potentially protective factor in the genital mucosa and draining lymphoid tissue.

Gp120 is reported to directly stimulate the production of PGs. Rats injected intracerebroventricularly with gp120 show increased expression of COX-2 in the brain cortex and increased levels of PGE_2 in whole-brain homogenates. Indomethacin administration abolishes PGE_2 rises and the accompanying apoptosis of cortex cells (Bagetta *et al.*, 1998). Cultured neuroblastoma cells also up-regulate the PGE-synthesising arachidonic acid cascade and undergo apoptosis in response to gp120 (Maccarrone *et al.*, 1998). They can be rescued from this death by COX inhibitors (Corasaniti *et al.*, 1995). Cultured astrocytoma cells release PGE_2 in response to culture with gp120 (Mollace *et al.*, 1994). None of the above reports of gp120 induced PG synthesis identifies the strain of HIV-1 from which the gp120 was derived or its cellular tropism. M- and T-tropic HIV-1 derived gp120 is reported to

stimulate phosphorylation of the CD4-p56^{lck} receptor signal transduction pathway but not PG formation in the THP-1 human monocytic cell line (Hui *et al.*, 1995). In primary bronchoalveolar lavage macrophages however, gp120$_{IIIB}$ induced a substantial PGE_2 secretion. This was blocked by indomethacin (Denis, 1994). Gp120$_{IIIB}$-induced PGE_2 production enhanced the growth of an AIDS-associated strain of *Mycobacterium avium* in gp120-treated macrophages (Denis, 1994).

Prostaglandin signals may be linked to the Notch pathway via NF-κB. Activated NF-κB can trigger the expression of Notch ligand (Bash *et al.*, 1999), and PGs can influence the activation state of NF-κB (Conte *et al.*, 1997; D'Acquisto *et al.*, 1998; Rossi *et al.*, 2000), although whether PGs are inhibitory or activatory of NF-κB is not clear.

Summary of tolerance signals

In addition to Notch, antigen presentation in the context of several other signalling molecules may induce specific tolerance. The tolerogenic signal can come from suppressor T-cells or APCs.

IL-10 acts as a growth factor for the suppressor $CD4^+$ T-cells able to protect against IBD, and IL-10 knockout mice are unable to produce this protective cell population (Asseman and Powrie, 1998; Fowler and Powrie, 1999; Leach *et al.*, 1999). IL-10 producing suppressor T-cells are induced in models of oral tolerance (Krause *et al.*, 2000). PGE, 19HO-PGE and seminal plasma can induce IL-10 production from whole blood (Kelly *et al.*, 1997a). In the eye, lymphoid cells undergoing Fas-mediated apoptosis produce IL-10 before dying in order to limit the immune response to their self-antigens (Gao *et al.*, 1998). As well as being anti-proliferative to $CD8^+$ T-cells, IL-10 down regulates macrophage endocytosis (Montaner *et al.*, 1999). Reports of the effects of HIV-1 infection and incubation with gp120 on monocyte / macrophage production of IL-10 are contradictory. Some authors observe no change in either basal or LPS-stimulated release of IL-10 in response to HIV-1 infection or the presence of gp120 (DeReuddreBosquet *et al.*, 1997; Bergamini *et al.*, 1998). Other reports describe an increased expression of IL-10 by macrophages and macrophage / T-cell syncytia after HIV-1 infection or

incubation with gp120 (Gessani *et al.*, 1997; Taoufik *et al.*, 1997; Hammond *et al.*, 1998). It seems likely that the maturation state of the monocyte derived macrophages is important in determining the extent of the IL-10 response. HIV-1-induced reductions of macrophage secreted IL-12 may be the result of inhibition of IL-12 production by IL-10 Taoufik *et al.*, 1997). The outcome of HIV-1-induced IL-10 production is complex; immune-inhibitory properties of IL-10, and CCR-5 up-regulation in response to the cytokine (Houle *et al.*, 1999) would be expected to favour viral infection and replication. However, IL-10 acts on infected macrophages to slow the rate of viral replication, possibly by inhibiting complete macrophage maturation (Chang *et al.*, 1996).

TGF-β is produced by the regulatory T-cells providing protection from disease in models of IBD. Neutralising antibodies to TGF-β can ablate the suppressor cells' protective effect (Powrie *et al.*, 1996). Interestingly, in TGF-β knockout mice, a protective suppressor T-cell population can still develop (Barone *et al.*, 1998). When human macrophages ingest apoptotic cells, they produce TGF-β, presumably to avoid an inflammatory response (McDonald *et al.*, 1999). In the context of HIV-1, Tat has been demonstrated to induce TGF-β production by macrophages (Reinhold *et al.*, 1999; Rubartelli *et al.*, 1999). The role of PGs in tolerance has already been reviewed above. Other proposed mechanisms of tolerance induction include the macrophage cell surface expression of a tryptophan metabolising enzyme in order to prevent lymphocyte proliferation by starving them of a necessary extracellular precursor molecule (Mellor and Munn, 1999; Mellor and Munn, 2000), and the removal or reduction in production of pro-response cytokines such as IL-2 (Lombardi *et al.*, 1994; Lombardi *et al.*, 1995) and IL-12 (Taoufik *et al.*, 1997).

The hypothesis that Notch signalling is involved in tolerance induction does not preclude a role for any other molecule. There is a precedent for Notch signals to interact with other signals, including cytokine signals, and several components of the Notch pathway are common with other signalling pathways (see above). There has been little work examining the interaction of conventional tolerance signals with the Notch pathway, except that IL-10 has been shown to increase *notch-1* transcription in $CD4^+$ T-cells, and TGF-β up-regulate *serrate-1* mRNA in DCs and B-cells (Lamb *et al.*, 2000). Although experimental systems involving transfection of cells to express

high levels of Notch ligands result in tolerance induction and point to a way of inducing tolerance therapeutically, it seems likely that in physiological situations no one signal will be overriding. The decision that the immune system makes when it decides between tolerance or immunity is a vital one, and one would expect it to be made in the light of all the available information. Notch signals may help naive T-cells to adopt a regulatory cell fate. Furthermore, Notch signals may be used to allow regulatory T-cells to regulate the activity of other T-cells.

Notch experimental difficulties

The proteins of the Notch signalling pathway are highly conserved between species, so it is difficult to raise antibodies to them for experimental use. A handful of rather poor affinity antibodies are now available, but most of these have been raised against the cytoplasmic region of the proteins. This precludes their use in live cells and makes it difficult to use them in flow cytometry. The University of Edinburgh's Centre for Inflammation Research has had some success using an anti-Serrate antibody in immunohistochemistry on Cytospins and tissue section.

Real-time PCR studies avoid the need to use antibodies and this approach has been taken to the Notch pathway in this thesis. Results based on measured mRNA levels need to be interpreted with caution, translational regulation can mean that the level of a specific mRNA does not always vary in line with its protein (Lie and MacDonald, 1999; McDonald *et al.*, 1999). Nevertheless, there is a precedent at the Centre for Inflammation Research and other centres for using Real-time PCR to study the Notch and related pathways and this supports the contention that the Notch pathway is principally regulated at the transcription level. Notch is cleaved upon activation (Kidd *et al.*, 1998; Schweisguth, 2000), so signalling through Notch is proceeded by an up-regulation of *notch* mRNA to replenish cell surface Notch. There is also data (Smith *et al.*, 1995; Robey *et al.*, 1996) which suggest that one of the transcriptional targets of Notch is *notch* itself. Whether each *notch* locus is specifically up-regulated in response to activation of its corresponding protein, or all available *notch* genes are expressed in response to Notch activation is not known. Notch cleavage releases the intracellular domain of Notch. This portion of the receptor is believed to enter the nucleus and promote transcription of *HES* and *deltex*

by interacting with DNA-binding proteins (Schweisguth, 2000). Transcriptional activation of Notch target-genes is, therefore, a direct read-out of this signalling event.

Aims of chapter

Data presented in chapter 4 demonstrate that HIV-1 gp120 induces changes in APC cell-surface phenotype. This chapter investigates whether gp120-induced changes in phenotype are linked to changes in the functional ability of APCs and T-cells. The novel and exploratory nature of this work necessitated the investigation of a large number of molecular candidates in order to select those for further study rather than the investigation a more defined area with greater rigor.

Specific questions

- Does gp120 cause a defect in antigen presentation and T-cell stimulation by APCs?
- Could any defect be interpreted as gp120 induced tolerance?
- Is the Notch pathway, prostaglandin or cytokine signalling involved in this tolerance induction?
- Which molecules of the Notch pathway are important in gp120 responses?
- Does seminal plasma induce tolerance via the Notch pathway in a similar manner to gp120?
- Does Notch signalling differ, functionally or molecularly, between T-cells, macrophages and DCs?

Methods

Refer to chapter 2 for details of materials and methods.

Cell culture

Macrophages. Monocytes were isolated from Buffy coats obtained from blood donations. All cells used in this chapter were homozygous wildtype for the *ccr5* locus (see chapter 3). Blood cells known to be positive for HLA DRB1*0101 were kindly donated by a colleague and found to be homozygous wildtype for the *ccr5Δ32* mutation. Monocytes were differentiated to macrophages over a period of 7 days and immuno-phenotyped before use as described in chapter 2.

Langerhans cells. DCs were generated from primary monocytes over a period of 7 days by culture with GM-CSF and IL-4 as described in chapter 2. To better model *in vitro* the LCs present in the genital mucosa (Purcell *et al.*, 1996; SaintAndreMarchal *et al.*, 1998), an LC-type phenotype was induced in all the DCs used in this chapter by addition of TNF-α one day before use of the cells (Strunk *et al.*, 1996).

T-cells. $CD4^+$ T-cells were isolated from PBMCs obtained from Buffy coats using a depleting cell-isolation column from R&D Systems. Cells were immuno-phenotyped to assess purity before use.

HA1.7 T-cell line. This cell line was passaged and stimulated with antigen on a 7-day cycle. Cells were taken for proliferation experiments at day 7 before re-stimulation to ensure that cells were quiescent at the start of the experiment.

HA1.7 and AC1.1 activation. This procedure was carried out by Dr Adrienne Verhoef (Department of Biology, Imperial College, London). Tissue culture plates were coated with anti-CD3 (10μg/ml) and anti-CD28 (1μg/ml) for 1 hour at room temperature. Plates were then washed and 1ml of HA1.7 or AC1.1 cells added at 2×10^6/ml for various times. RNA was extracted from these cells and sent to our lab on dry ice.

HA1.7 and AC1.1 anergy induction. This procedure was carried out by Dr Adrienne Verhoef. 2×10^6 HA1.7 or AC1.1 cells were incubated respectively with

25μg/ml of $HA^{306-318}$ or Der P II^{28-40} in 1ml of medium for various times. RNA was then extracted and sent to our lab on dry ice.

Cell treatments

Cultures were stimulated with the agents listed below, at the concentrations and for the times given in the results section of this chapter.

$Gp120_{IIIB}$ and **R5-gp120** were obtained from the NIBSC Centralised Facility for AIDS Reagents. These baculovirus expressed recombinant proteins were derived from the T-cell line adapted virus strain HIV-1_{IIIB} (Ratner *et al.*, 1985, GenBank accession number X01762) and from cDNA isolated from a primary macrophage of paediatric AIDS patient MN (Gurgo *et al.*, 1988, GenBank accession number U72495). The gene sequences used to generate both recombinant gp120s were kindly analysed according to published criteria (DeJong *et al.*, 1992; Fouchier *et al.*, 1992) by Dr Peter Simmonds (Laboratory for Clinical and Molecular Virology, University of Edinburgh) and confirmed to be X4-tropic and R5-tropic respectively.

3.7 peptide is a 44-mer incorporating discontinuous epitopes of gp120. It was obtained from Professor Robert Ramage (Department of Chemistry, University of Edinburgh). The sequence, design and synthesis of 3.7 is described in chapter 6.

FMDV peptide was also obtained from Professor Ramage and used as a control for 3.7. FMDV is a 44-mer peptide based on a sequence derived from a different virus (bovine Foot and Mouth Disease Virus) and like 3.7 has a C-X-C bond incorporated in its structure. There is no sequence homology between 3.7 and FMDV.

QS4120 anti-CD4. This mouse monoclonal antibody is directed against the CDR2 region of CD4 and inhibits gp120 / CD4 binding (Howie *et al.*, 1998).

Sheep IgG was isolated using a protein G column as described in chapter 2 from non-immunised sheep serum (SAPU). Having a molecular weigh of approximately 150kD but being unable to bind to human cells, it was used as a control for both gp120 and QS4120.

Seminal plasma extract (SPE), PGE_2 and 19HO-PGE_2 were obtained courtesy of Professor Rodney Kelly (MRC Centre for Reproductive Biology, Edinburgh).

Indomethacin, also obtained from Professor Kelly was added at 5μM to some cultures in order to block prostaglandin synthesis by inhibiting COX-2.

Cell harvesting and analysis

Proliferation assays, flow cytometry, semi-quantitative RT-PCR, Real-time RT-PCR, prostaglandin determination, immunophenotyping and ELISA were carried out as described in chapter 2. Vivien Grant (MRC Centre for Reproductive Biology, Edinburgh) carried out prostaglandin determination. Real-time RT-PCR was carried out in collaboration with Gail Baldie (MRC Centre for Reproductive Biology, Edinburgh). James Logie carried out the *IL-10* semi-quantitative RT-PCR, under my supervision as part of his BSc Experimental Pathology final year project.

Results

Cell purities.

Monocyte derived macrophages and LCs, and column purified $CD4^+$ T-cells were phenotyped by flow cytometry in order to assess their purity. Macrophage cultures were >90% MHC class II^+, >80% $CD14^+$, >85% $CD80^+$ with contaminating T-cells ($CD3^+$) of <10% and B-cells ($CD40^+$) of <0.5%. LC cultures were >90% $CD1a^+$, >85% $CD54^+$, >80% $CD80^+$ and >85% MHC class II^+ (>65% MHC class II^{hi}). Contaminating T-cells ($CD3^+$) were <3% and B-cells ($CD40^+$) <10%. Contaminating monocytes / macrophages ($CD14^+$) were <20%. Column isolated $CD4^+$ T-cell cultures were 98% $CD3^+$, 93% $CD4^+$, 5% $CD8^+$, 0.25% $CD14^+$ (monocytes) and 2% $CD40^+$ (B-cells).

PCR standard curves

Figure 5.4 shows the data obtained from a variable cycle RT-PCR experiment for IL-10. 31 cycles were chosen for all future semi-quantitative RT-PCR reactions under similar conditions. The level of *IL-10* mRNA present in each sample was

expressed relative to the level of the *β-actin* housekeeping gene mRNA, determined as described in chapter 4.

The Notch pathway is involved in experimental T-cell line anergy induction

The antigen specific $CD4^+$ T-cell lines HA1.7 and AC1.1 were treated *in vitro* to render them anergic or activated. RNA was isolated from these cells at various post treatment time-points, and mRNA levels for genes of the Notch signalling pathway determined. Figure 5.5 shows relative levels of Notch pathway transcripts in HA1.7 cells. Figure 5.6 shows a smaller set of results from AC1.1 cells. Several mRNAs were up-regulated in HA1.7 cells in response to activation or anergy (figure 5.5). However, in general the initially elevated transcript levels in activated cells declined after about 24 hours, whereas specific mRNA levels in anergic cells continued to rise until at least 120 hours (5 days). Among the Notch ligands *serrate-2* and especially *delta-1* transcripts were most elevated in anergic cells. There is some indication that *notch-1, -2* and *-4* mRNA levels may be higher in anergic as opposed to activated cells. With regard to mRNA for intracellular signalling proteins acting down-stream of Notch, *deltex* levels remained low and unchanged in all samples, and *HES-1* was not detectable in any of the HA1.7 cells (not shown). The picture in AC1.1 cells is less complete (figure 5.6), but broadly in agreement with the HA1.7 results, showing *delta-1* mRNA levels varying the most and *deltex* mRNA remaining constant. *HES-1* mRNA was also absent from AC1.1 cells (not shown).

Inhibition of HA1.7 antigen specific proliferation

In order to investigate if gp120 and other agents were able to induce HA1.7 anergy, HA1.7 cells were incubated with gp120 or anti-CD4 before being driven to proliferate by macrophage presentation of $HA^{306\text{-}318}$ peptide. Pre-treatment of HA1.7 cells did not result in a significant reduction in basal proliferation in the absence of antigen (figure 5.7b), or proliferation in response to 0.5μg/ml (figure 5.7d) or 1μg/ml (figure 5.7f) of $HA^{306\text{-}318}$.

However, if macrophages were pre-treated with R5-tropic gp120 or anti-CD4, but not $gp120_{IIIB}$ or sheep IgG, their ability to support antigen specific HA1.7

proliferation was reduced (figure 5.7a and c). This reduction in HA1.7 proliferation was not seen when $HA^{306-318}$ was added at its highest dose of 1μg/ml (figure 5.7e).

Pre-treatment of LCs for 48 hours with various agents also caused a reduction in their ability to support HA1.7 proliferation. R5-tropic gp120, anti-CD4, PGE_2 and SPE all caused a significant reduction in LC ability to support HA1.7 proliferation in response to 0.2μg/ml (figure 5.8a) or 0.5μg/ml (figure 5.8b) of $HA^{306-318}$. The control protein sheep IgG did not affect proliferation, nor did $gp120_{IIIB}$. SPE produced the most profound suppression of proliferation. The presence of indomethacin abrogated the suppression caused by R5-tropic gp120 and anti-CD4.

Gp120 treatment does not cause macrophage apoptosis

In order to investigate the possibility that gp120 and anti-CD4 treatment of macrophages lead to the reduced ability of APCs to support T-cell proliferation by causing APC apoptosis, macrophages were assayed for apoptosis by the annexin V method following various treatments. R5-tropic gp120, $gp120_{IIIB}$ and anti-CD4 treatment for 48 hours did not induce detectable increases in macrophage apoptosis above the level present in control cells treated with sheep IgG. Figure 5.9 shows a background level of 7 to 9% apoptotic cells in macrophages treated with sheep IgG, gp120 or anti-CD4. To confirm the ability of the annexin V method to detect apoptosis, macrophages were starved of medium for 48 hours; this resulted in over 95% of cells becoming apoptotic or secondarily necrotic (figure 5.9a).

Gp120 can induce serrate *mRNA in $CD4^+$ T-cells*

In order to investigate the possible role of the Notch pathway genes (induced in anergic HA1.7 T-cells) in primary $CD4^+$ T-cell responses to gp120 treatment, various concentrations of gp120 or anti-CD4 were incubated with purified $CD4^+$ T-cells. Real-time PCR showed an up-regulation of *serrate-2* mRNA in response to R5-tropic gp120 and anti-CD4 (figure 5.10). These increases in *serrate-2* mRNA were dose dependent. The increase in *serrate-2* mRNA in response to $gp120_{IIIB}$ was less certain. No consistent changes in mRNA for *notch-1*, *delta-1* or *deltex* were observed. In

common with HA1.7 and AC1.1 T-cells, *HES-1* was not detectable in primary $CD4^+$ T-cells.

The Notch pathway may be involved in reduced macrophage and LC capacity to support proliferation

Figure 5.7 and 5.8 show that treatment of APCs with gp120, SPE or PGE_2 reduced their capacity to support T-cell proliferation. In order to investigate the possible involvement of the Notch pathway in this phenomenon, mRNA levels of Notch pathway genes were measured following APC treatment with various agents.

Notch ligands. Following 6 or 48 hours of LC treatment with R5-tropic gp120, $gp120_{IIIB}$, SPE or PGE_2, *serrate-1* and *-2* mRNA levels showed some variation but no consistent pattern of change (figure 5.11a, b and c). However, *delta-1* mRNA levels at 6 hours (figure 5.11d) increased in response to treatment with all four agents. There was no apparent difference between responses to R5-tropic gp120 and $gp120_{IIIB}$. Gp120-induced *delta-1* mRNA increases were partly abrogated by the presence of indomethacin. By 48 hours *delta-1* mRNA levels had largely returned to basal levels. On macrophages convincing *delta-1* mRNA increases were not observed (figure 5.12a and b).

Notch receptors. In general no LC treatments resulted in dramatic *notch* mRNA increases. A small dose dependent increase in *notch-1* in response to PGE_2 (figure 5.11g), and an increase in *notch-2* in response to $gp120_{IIIB}$ are exceptions to this observation. In macrophages 48 hours of treatment with SPE caused large increases in *notch-1* mRNA (figure 5.12d).

Down-stream signalling molecules. *HES-1* and *deltex* mRNA was detected in LCs, but not in macrophages. A wider survey of *HES-1* and *deltex* expression showed that both mRNAs were detected in monocyte-derived LCs from donors A and B, but were absent from monocyte-derived macrophages from donors B, C and D (data not shown).

In LCs gp120-, SPE- and PGE_2-induced changes in *HES-1* and/or *deltex* mRNA levels were larger at 48 hours than 6 hours (this is in contrast to Notch ligand mRNA increases, which were greatest at 6 hours). *HES-1* mRNA expression was induced in LCs at 48 hours in a dose dependent fashion (figure 5.12m). There was no

apparent strain specificity of gp120-induced effects. Little change in *HES-1* mRNA level was seen at 48 hours in response to SPE or PGE_2, or at 6 hours in response to any agent. In LCs , all four agents induced an increase in *deltex* mRNA (figure 5.11n and o). This increase was greatest at 48 hours, dose dependent, and in the case of gp120, non-strain specific and partly abrogated by indomethacin.

Peptide 3.7 may be able to mimic some of gp120's actions

The gp120-based peptide 3.7 (discussed in detail in chapter 6) could potentially mimic the effects of gp120 and modulate mRNA levels of Notch pathway genes. In order to investigate this possibility, 3.7 and an irrelevant (FMDV) peptide were incubated with LCs for 6 and 48 hours. By 6 hours 3.7 induced an increase in *delta-1* (figure 5.13a) and *notch-1* (figure 5.13b) mRNA. Responses to 3.7 at 48 hours or changes to *HES-1* (figure 5.13c) or *deltex* (figure 5.13d) mRNA levels were no larger than those seen with FMDV peptide.

Prostaglandin may mediate gp120 effects

The observations that responses to gp120 may be partly abrogated by the addition of the COX-2 inhibitor indomethacin, and that some of the responses to gp120 are similar to those induced by PGE_2, and published reports of gp120-induced prostaglandin synthesis by microglia (e.g., Denis, 1994), suggest that the gp120 effects on Notch pathway mRNA may be mediated via prostaglandin production. In order to explore this possibility further, PGE_2 was measured in the supernatants of LC and macrophage cultures treated with gp120 for 6 and 48 hours (the same cells in which mRNA levels were measured). Both $gp120_{IIIB}$ and R5-tropic gp120 induced PGE_2 release by LCs at 6 hours (figure 5.14). By 48 hours this PGE_2 was no longer detectable in the supernatant. In macrophages gp120 seemed less able to induce PGE_2 release at the time-points assayed. The apparent PGE_2 release by macrophages in response to 1μg/ml of gp120 at 6 hours (figure 5.14) may have been caused by a single assay contamination event.

The effects of gp120 and PG on LC *HES-1* transcript induction were not identical. Both M-tropic and T-tropic gp120 induced *HES-1* mRNA at 48 hours

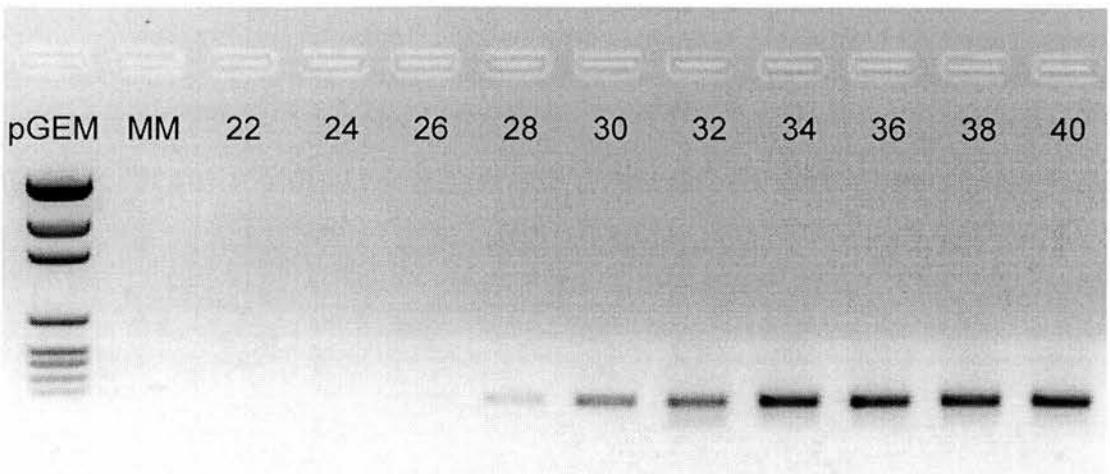
(figure 5.11m). This *HES-1* induction was abolished by indomethacin, suggesting that PG mediated it. However 6 or 48 hours of PGE_2 treatment did not result in a similar *HES-1* induction (figure 5.11l and m), despite similar effects of PGE_2 and gp120 on the induction of Notch ligand mRNA. It appears that PG synthesis (COX-2 activity) is required to mediate gp120-induced *HES-1* up-regulation, but that the effect may not be mediated solely by PGE_2-induced up-regulation of Notch ligand. Other signals from, or induced by, gp120 (for example CD4 ligation or non-PGE_2 PGs) may also be required. Alternatively, PGE_2 treatment of LCs *may* cause *HES-1* up-regulation at a time point not assayed in these experiments.

In addition to the Notch pathway, cytokines may be involved in APC tolerance induction

As well as the Notch pathway many other APC-derived signals are involved in tolerance induction. In order to investigate the possibility that cytokines may have been involved in the inhibition of HA1.7 proliferation seen in figures 5.7 and 5.8 (in addition to or in place of Notch signals), ELISAs were used to assay TGF-β1, IL-10 and IL-12 in APC supernatants. Selected supernatants from macrophage and LC cultures treated with gp120, SPE, PGE_2 or LPS for 6 or 48 hours (the same cultures in which mRNA levels were measured) were assayed for IL-10, IL-12 and TGF-β1. IL-10 and IL-12 were undetectable in all samples (except the LPS-treated positive controls, data not shown, limit of detection approximately 50pg/ml). However, TGF-β1 was detected in the supernatant of both macrophages and LCs at both 6 and 48 hours following treatment with SPE, PGE_2 and gp120 (figure 5.15). TGF-β1 release in response to gp120 was dose dependent (optimum concentration ~0.1μg/ml) and non-strain specific.

Because IL-10 production in response to gp120 has been previously reported (Borghi *et al.*, 1995), the failure to detect IL-10 by ELISA was of concern. *IL-10* mRNA was therefore measured by semi-quantitative RT-PCR (figure 5.16) in macrophages treated with gp120 for up to 96 hours. Although $gp120_{IIIB}$ and R5-tropic gp120 caused an up-regulation of *IL-10* mRNA (and presumably subsequent cytokine release) by 72 and 96 hours, gp120 caused an initial decline in *IL-10* mRNA

which may account for the failure to detect IL-10 protein by ELISA at 6 and 48 hours.



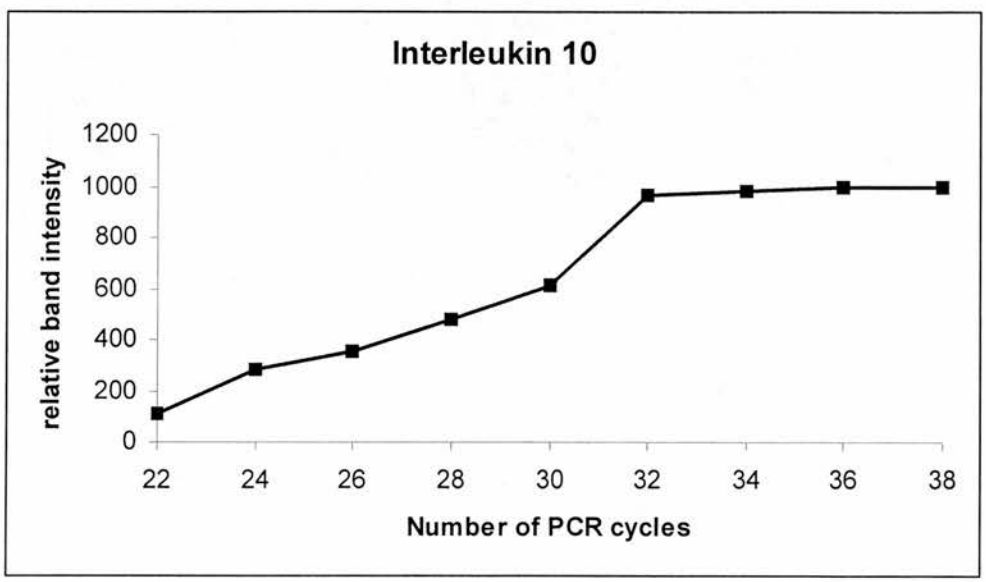


Figure 5.4. IL-10 standard curve. The electropherogram shows RT-PCR IL-10 product bands (328bp) obtained by amplification of 0.1pg of starting RNA for the number of cycles indicated. It can be seen that with increasing cycle number, the band intensity increases and then plateaus. The accompanying graph shows results of image analysis of the electropherogram.

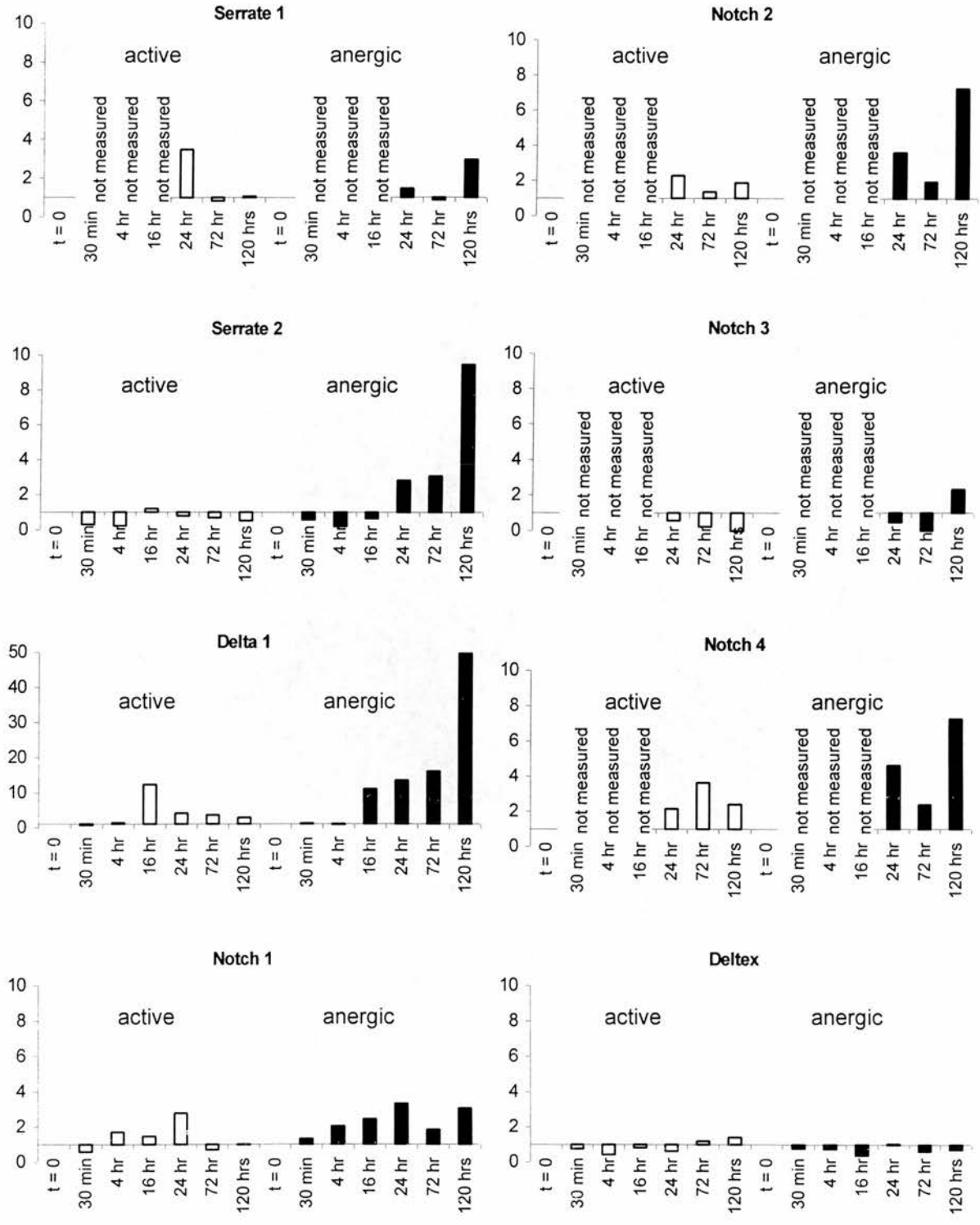


Figure 5.5. mRNA levels in activated and anergised HA1.7 T-cells. Cells were activated (white bars) or anergised (black bars) *in vitro*. Levels of specific mRNAs extracted from these cells were measured by real-time PCR, and are expressed relative to basal levels in un-stimulated cells (t = 0) given a value of 1. Graphs show mean mRNA levels from two experiments.

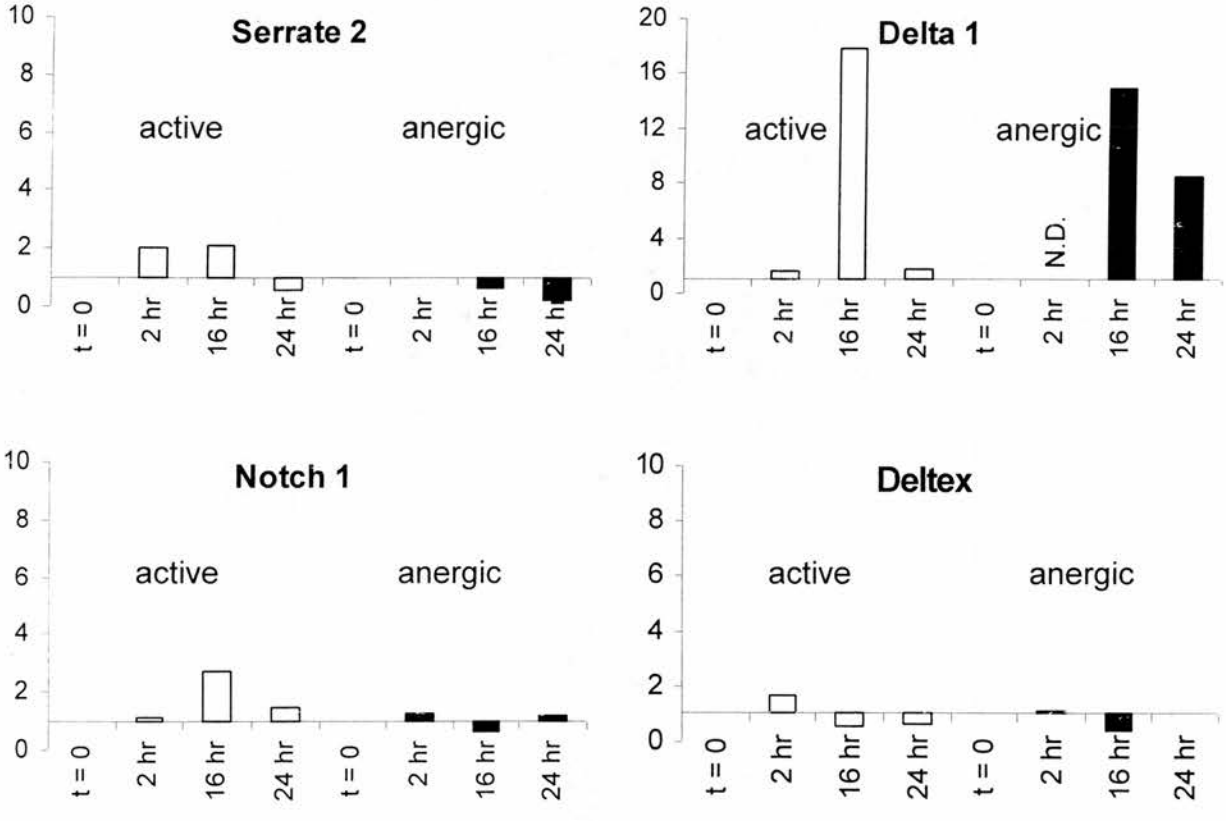
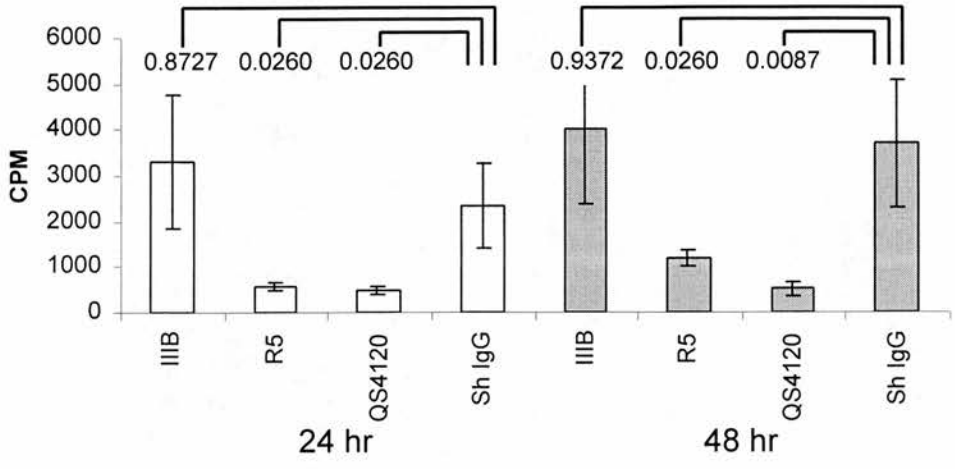
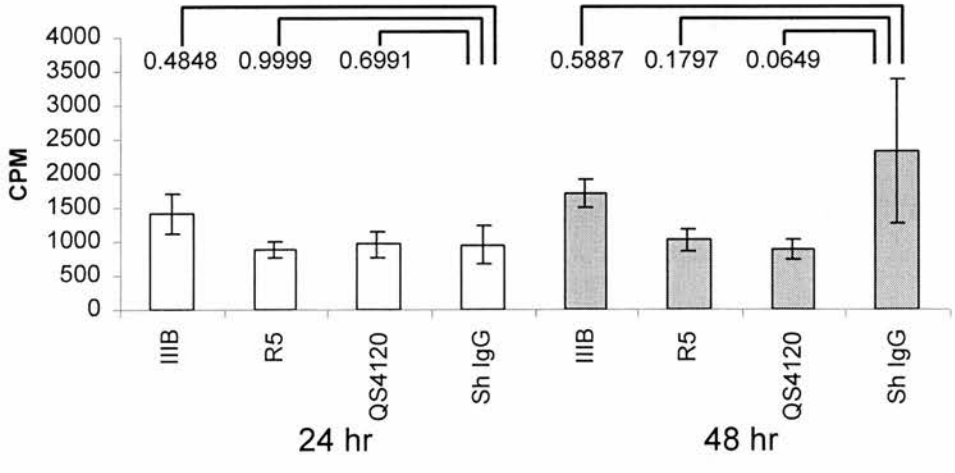


Figure 5.6. mRNA levels in activated and anergised AC1.1 T-cells. Cells were activated (white bars) or anergised (black bars) *in vitro*. Levels of specific mRNAs extracted from these cells were measured by real-time PCR, and are expressed relative to basal levels in un-stimulated cells (t = 0) which were given a value of 1. Graphs show mRNA levels from a single experiment. N.D. = not determined.

a) **MPh pretreated. Response to nil HA peptide**



b) **T-cells pretreated. Response to nil HA peptide**



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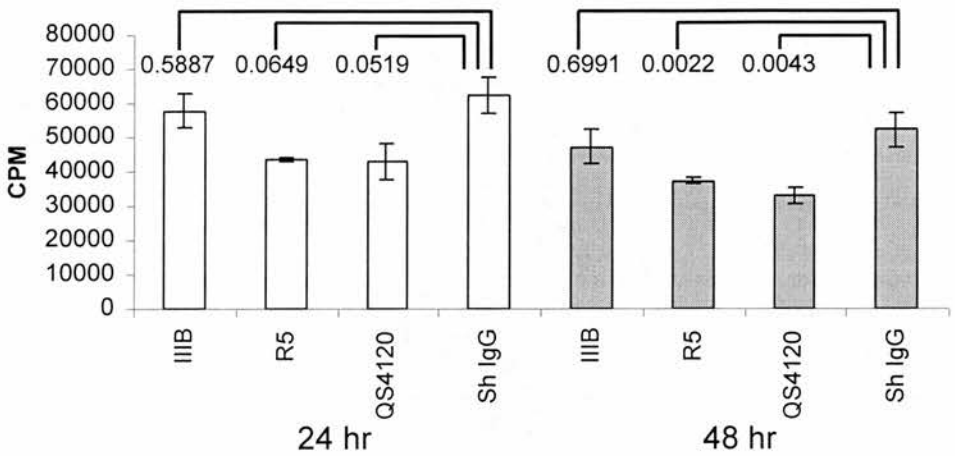
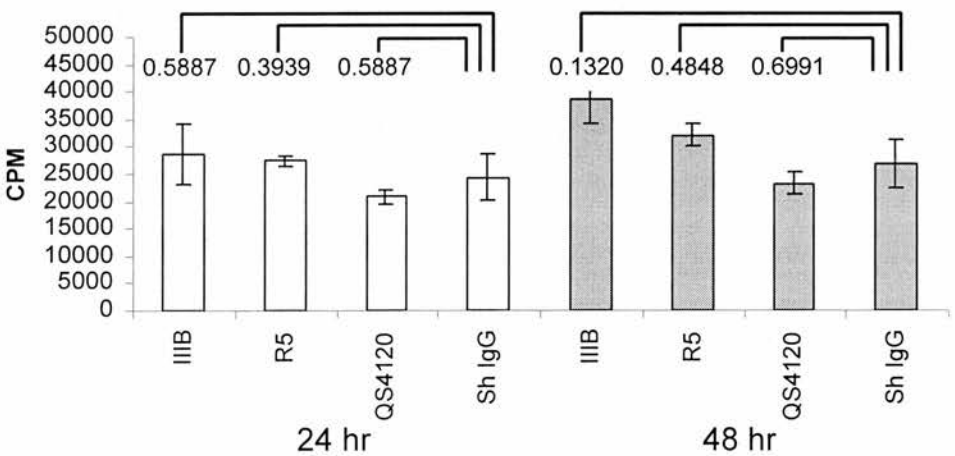
c) **MPh pretreated. Response to 0.5ug/ml HA peptide**



d) **T-cells pretreated. Response to 0.5ug/ml HA pept'**



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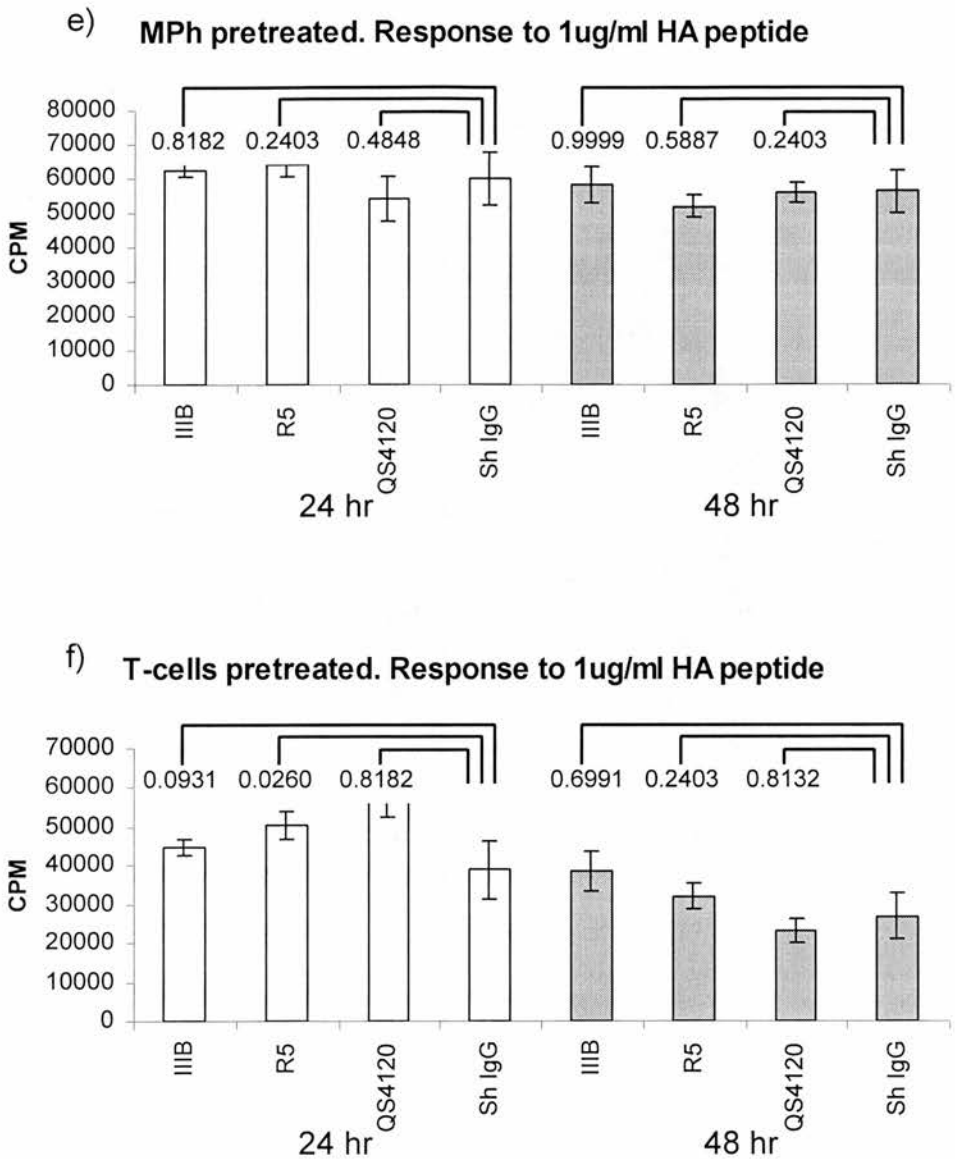
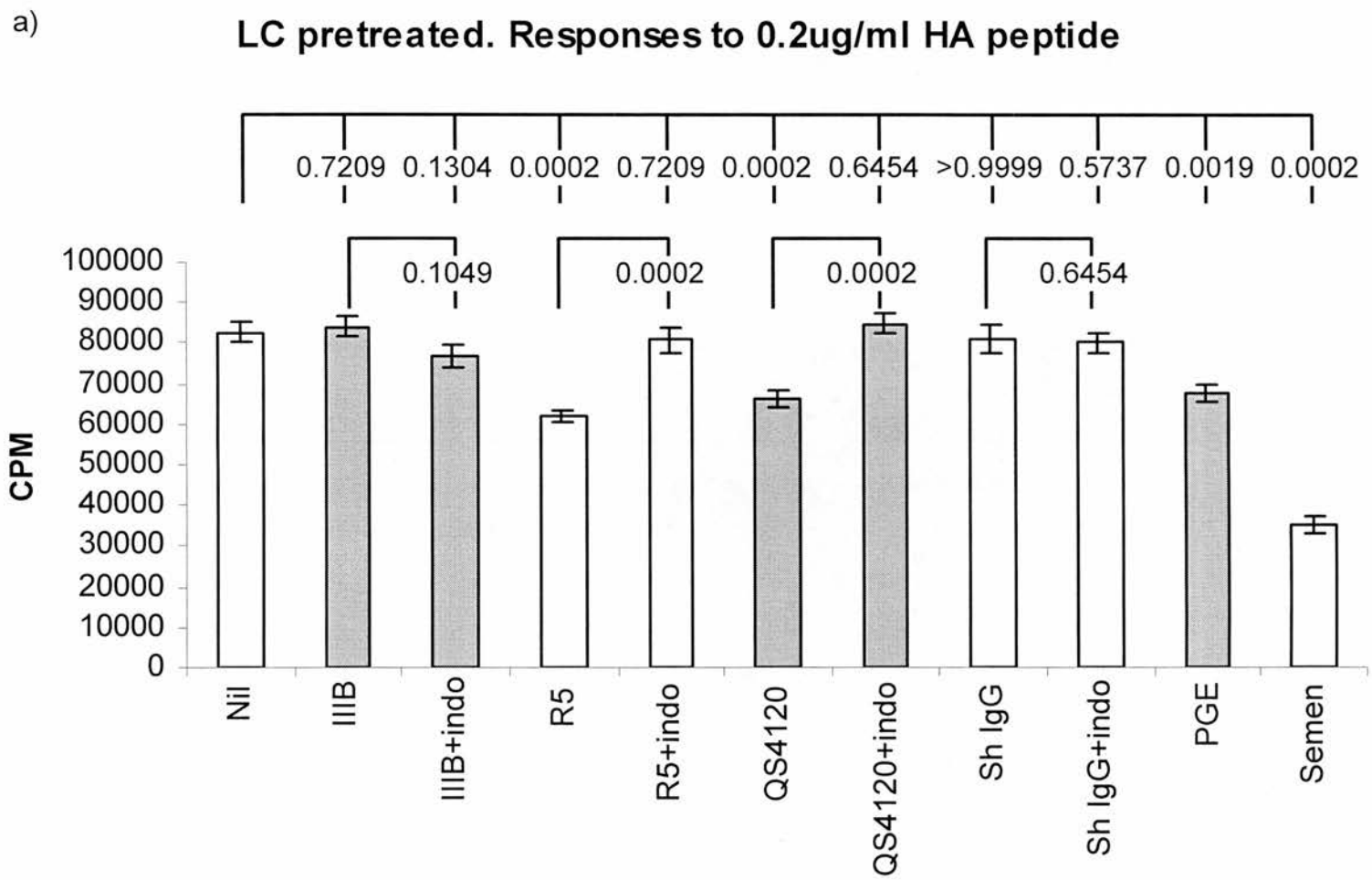


Figure 5.7. Antigen specific T-cell proliferation. Either macrophages or HA1.7 T-cells were pretreated with 0.1μg/ml of $gp120_{IIIB}$, R5-tropic gp120, anti-CD4 (QS4120) or sheep IgG (as a control) for 24 hours (white bars) or 48 hours (shaded bars). T-cells and macrophages were then mixed and antigen specific T-cell proliferation in response to various doses of $HA^{306-318}$ peptide was measured. P values (calculated by Mann-Whitney U tests) are shown comparing responses.

a)



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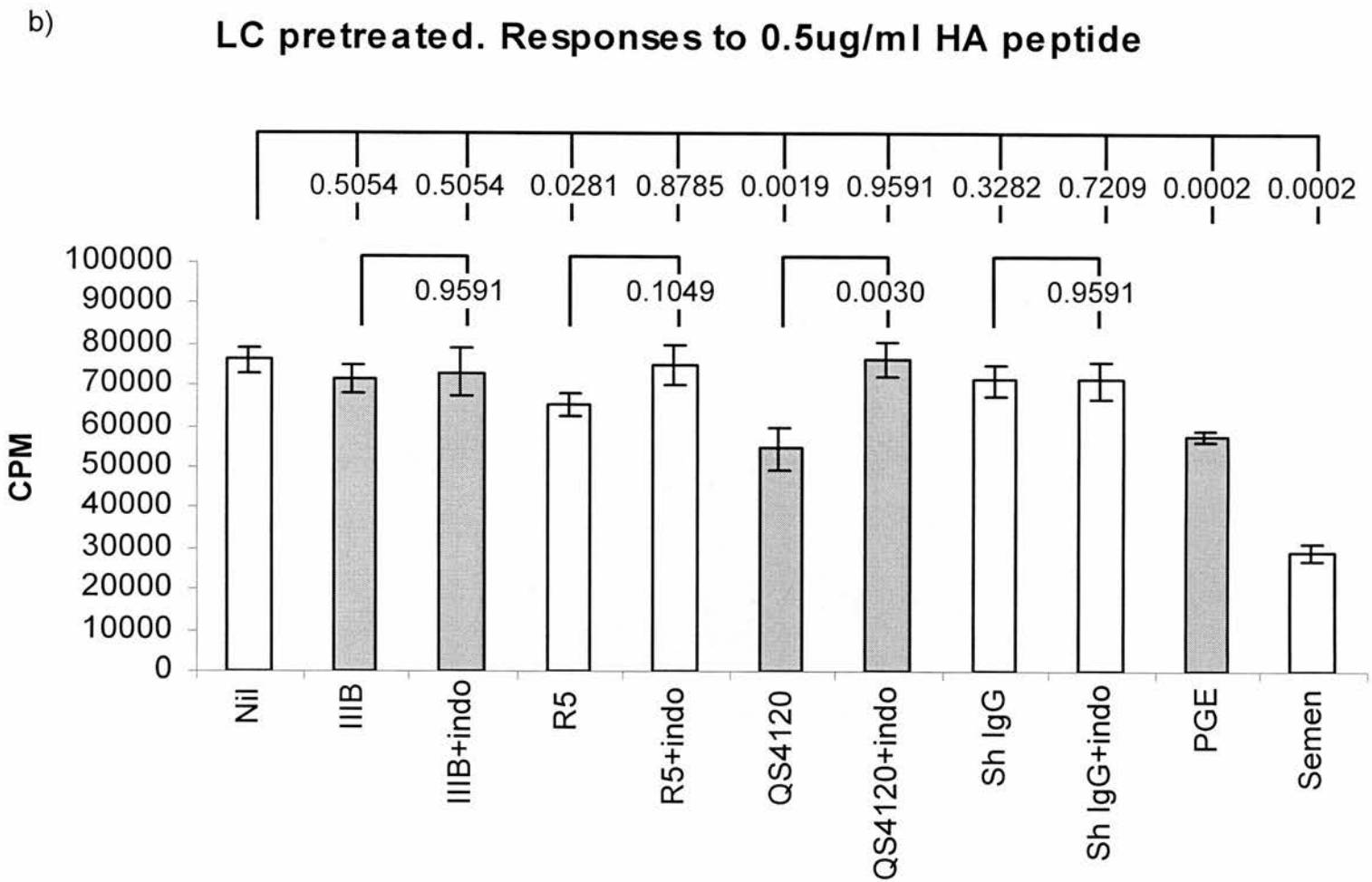
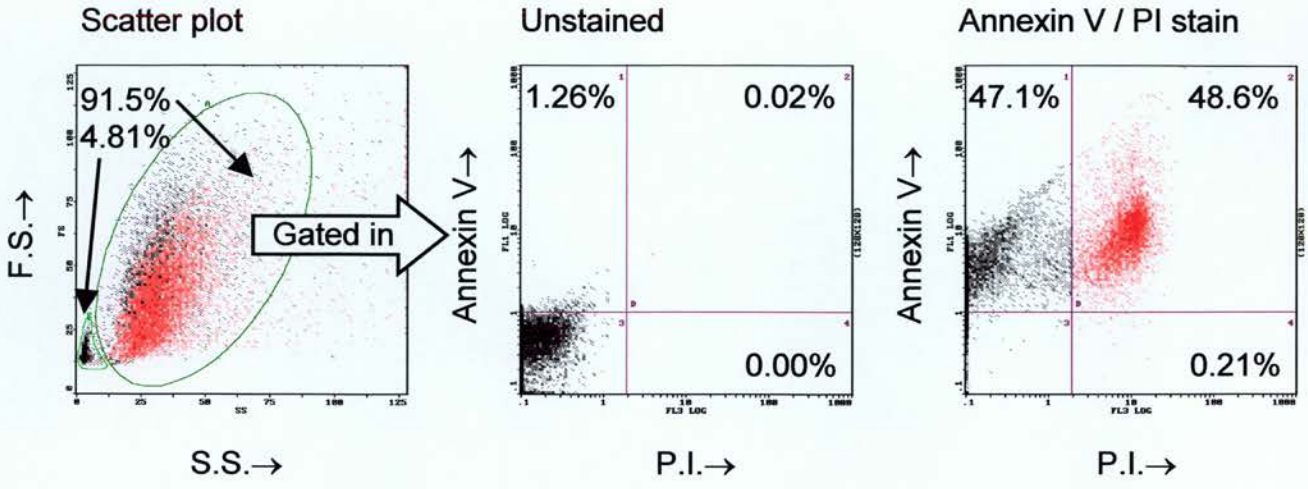
b)



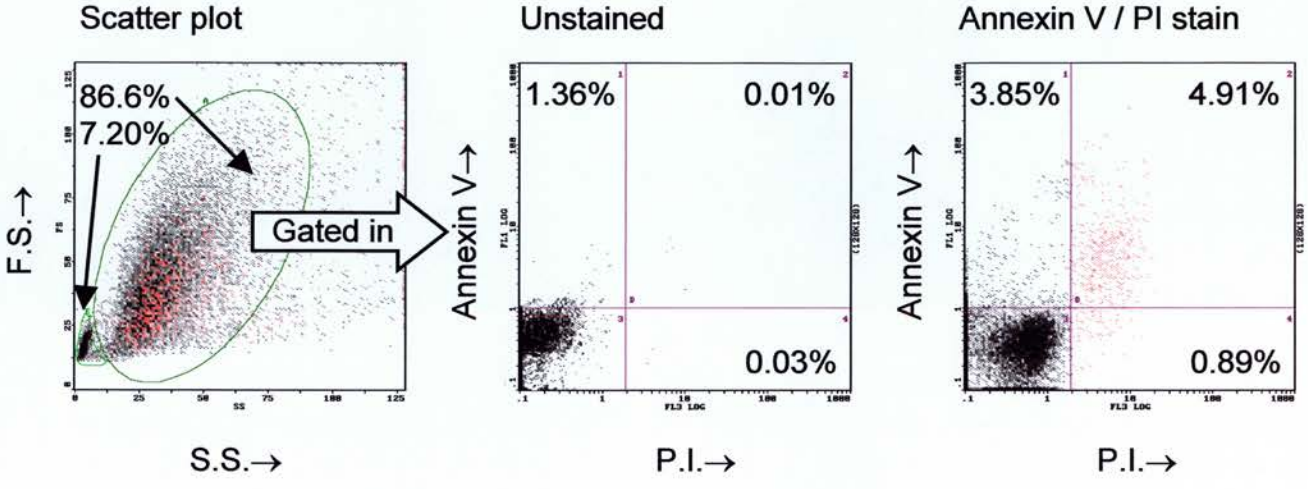
(caption over page)

Figure 5.8 (previous page). Antigen specific T-cell proliferation. LCs were pretreated for 48 hours with 0.1μg/ml of $gp120_{IIIB}$, R5-tropic gp120, anti-CD4 (QS4120) or sheep IgG, or with 1μM PGE_2, or with 0.05% seminal plasma extract, in the presence or absence of indomethacin (indo). HA1.7 T-cells were then added to the LCs, and antigen specific T-cell proliferation in response to 0.2 or 0.5μg/ml of $HA^{306-318}$ peptide was measured. P values (calculated by Mann-Whitney U tests) are shown comparing responses.

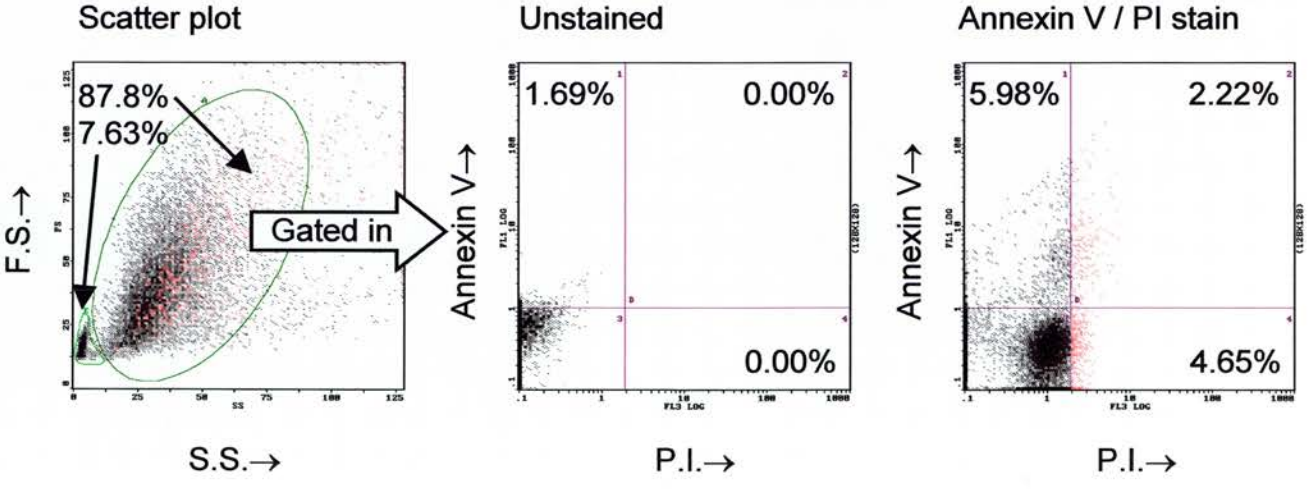
a) 48 hours - Medium withdrawal



b) 48 hours – Sheep IgG



c) 48 hours – R5-tropic gp120



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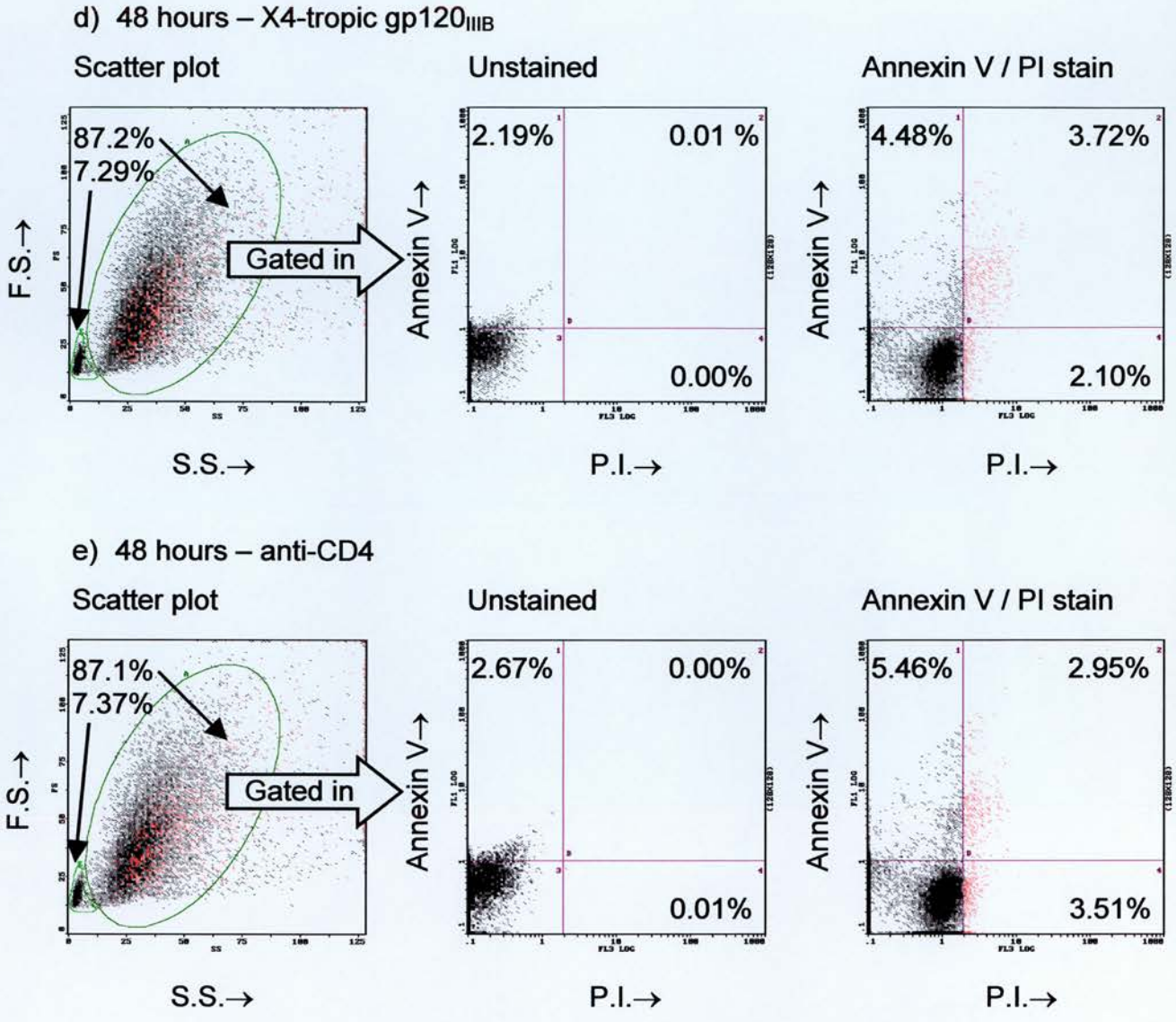


Figure 5.9. Annexin V macrophage apoptosis assay. Scatter plots show F.S. / S.S. characteristics of the cells and reveal a small (<8%) population of contaminating lymphocytes. Only those macrophages falling into the large scatter plot gate are shown in subsequent analysis. Part a) as a positive control macrophages were driven to apoptosis by culturing them in PBS in the absence of medium for 48 hours. Staining with P.I. and annexin V shows 95.7% of the cells as annexin V positive (i.e., apoptotic) and 48.6% showing secondary necrosis. Parts b), c), d), and e) show results from macrophages incubated for 48 hours with, respectively 1μg/ml of sheep IgG (control protein), R5-tropic gp120, $gp120_{IIIB}$ or anti-CD4 (QS4120).

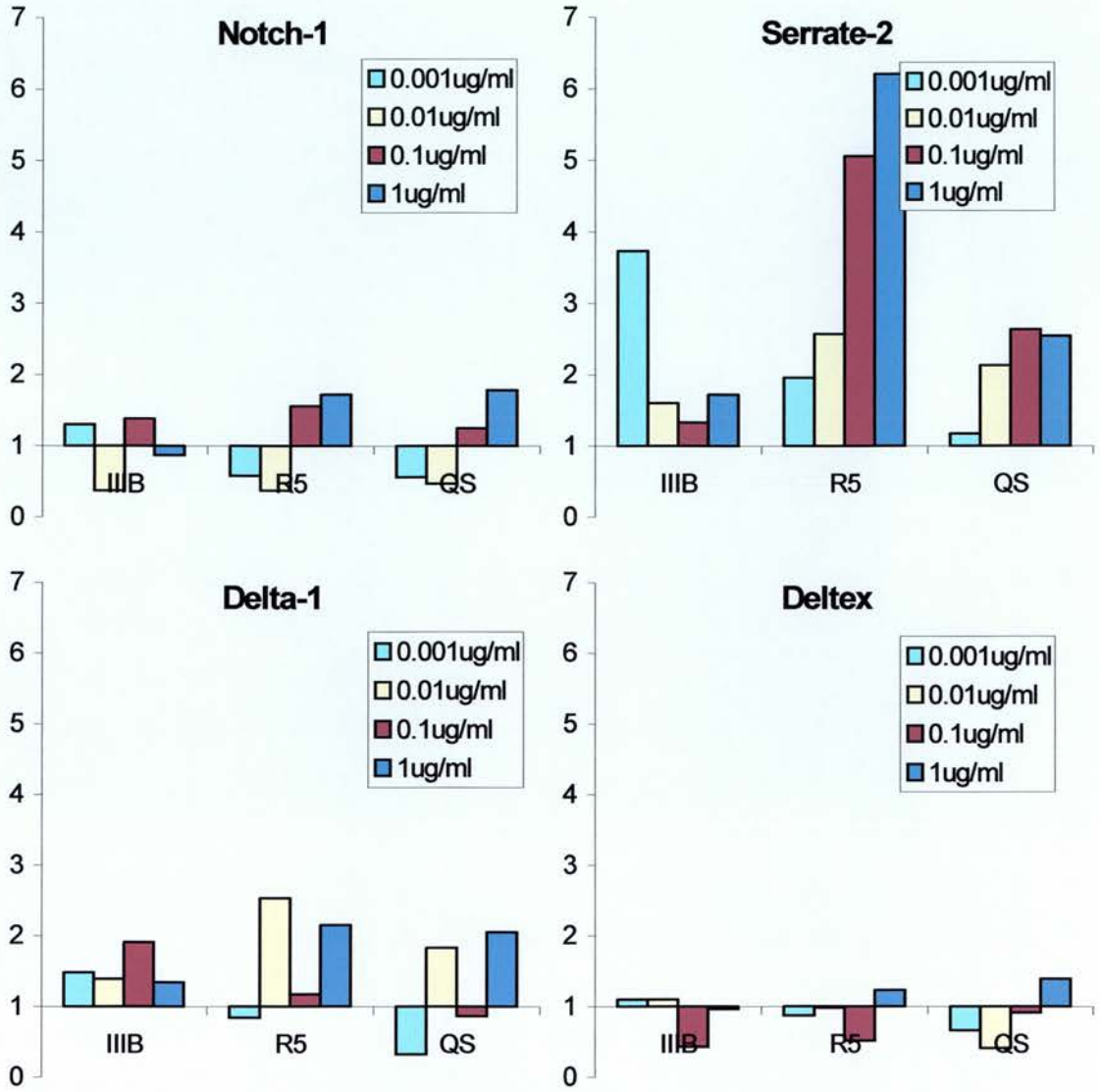
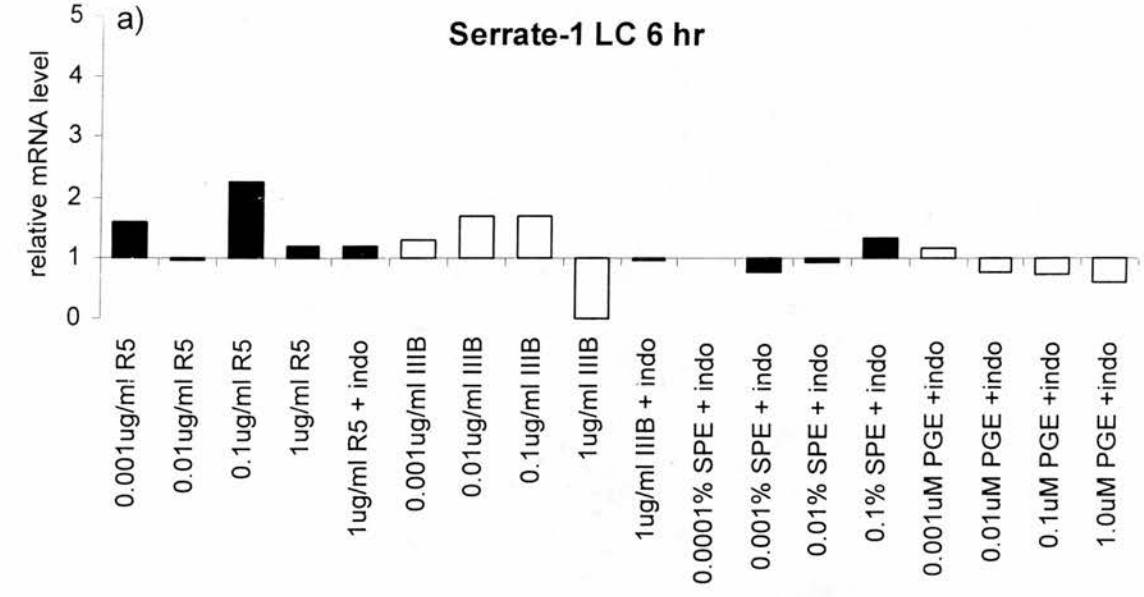
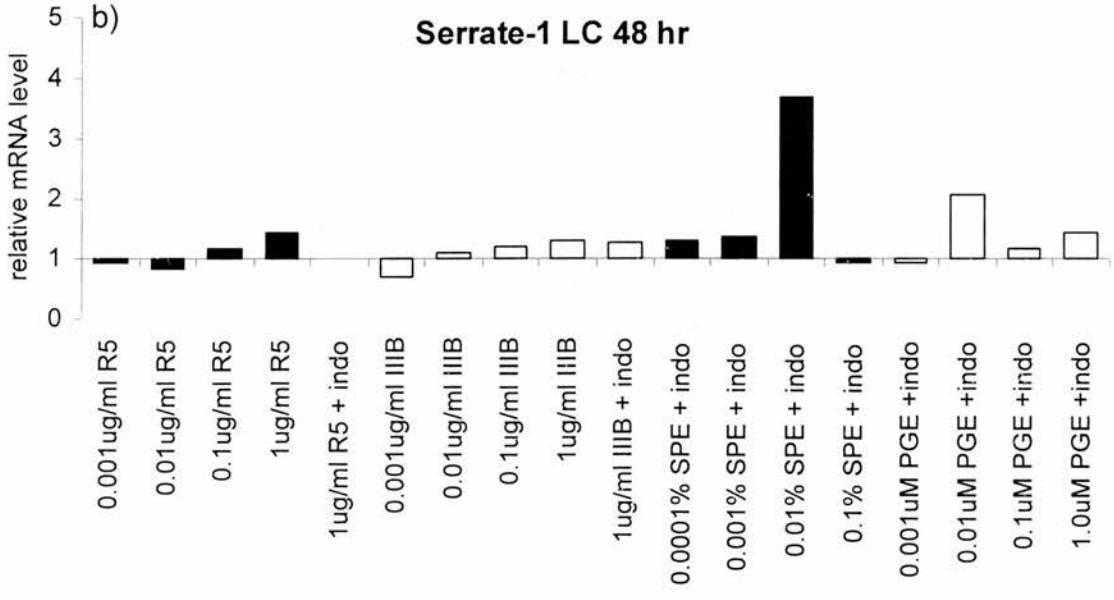


Figure 5.10. mRNA levels in primary CD4$^+$ T-cells. Primary CD4$^+$ T-cells were treated with various concentrations of $gp120_{IIIB}$, R5-tropic gp120 or cross-linking anti-CD4 (QS4120) for 48 hours. Levels of specific mRNAs extracted from these cells were measured by real-time PCR, and are expressed relative to basal levels in un-stimulated cells (given a value of 1).

N.B. Figure 5.11 follows. Caption and a simplifying summary table appear after the graphs





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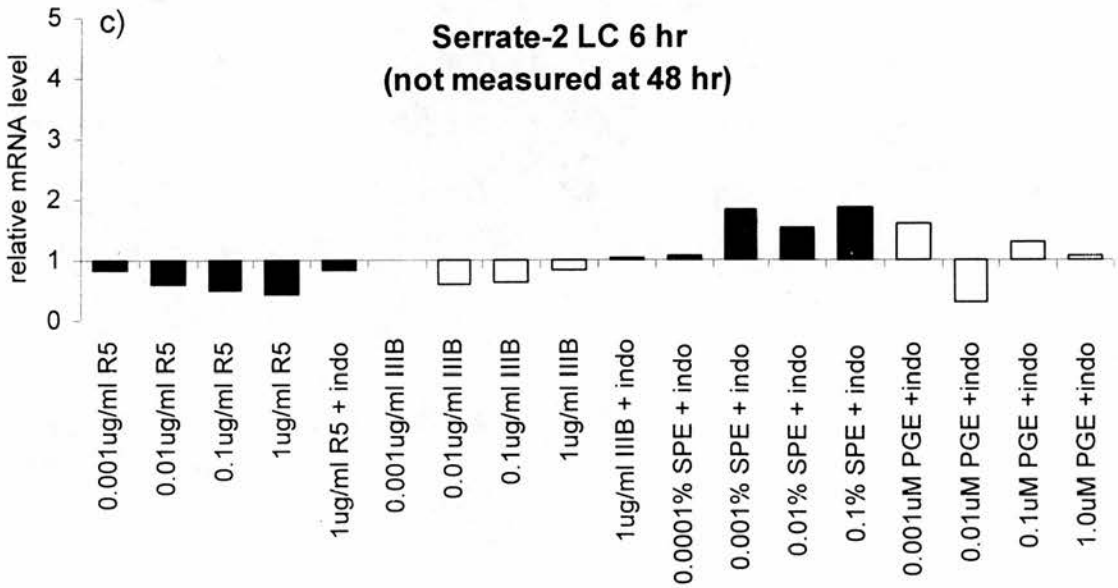

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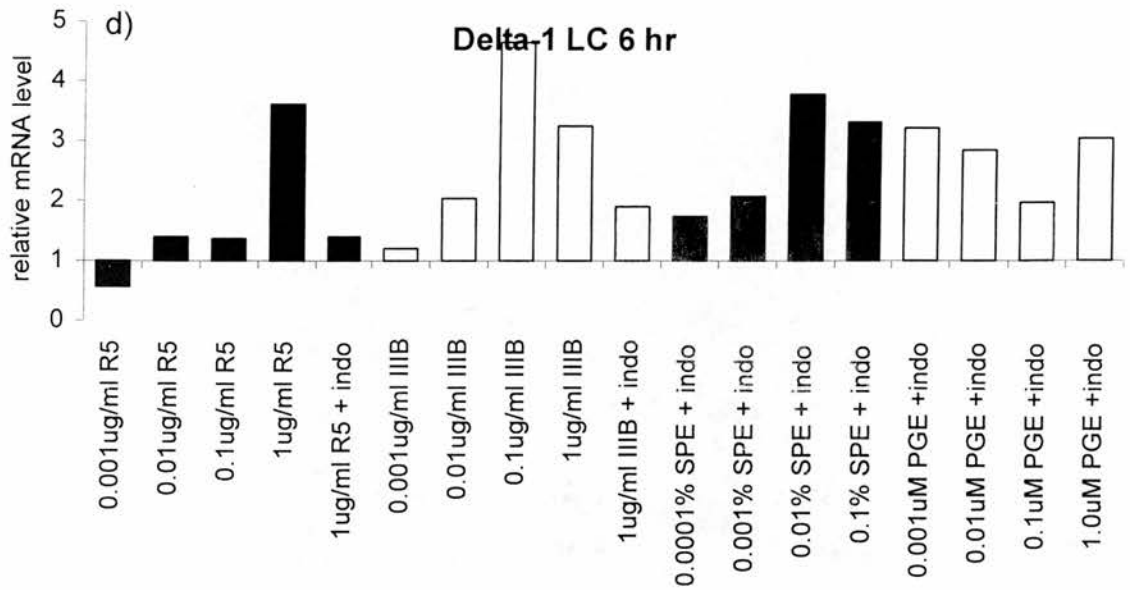

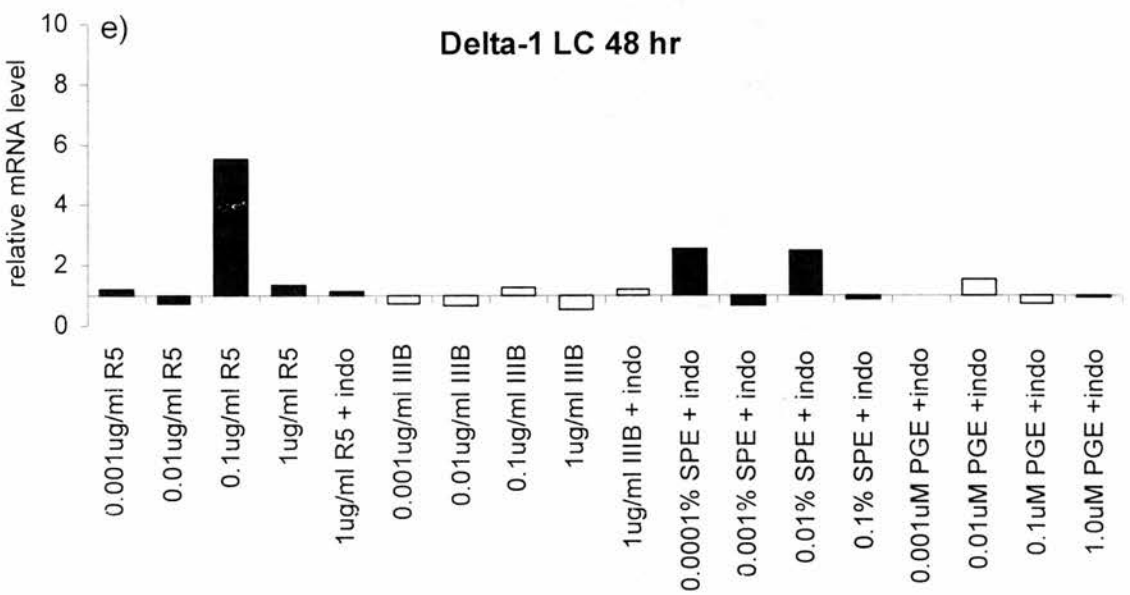

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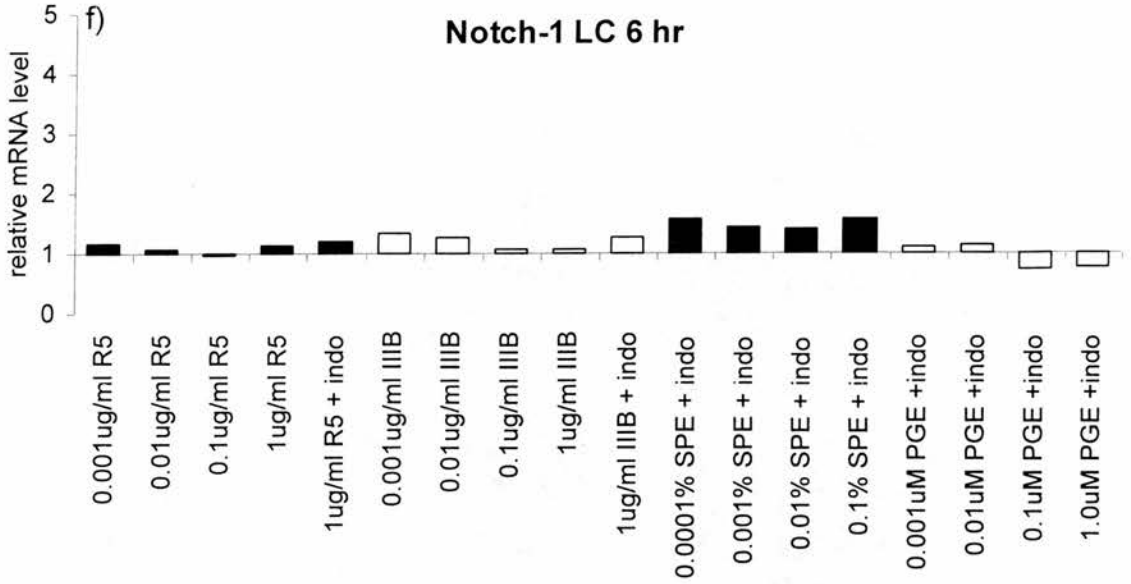

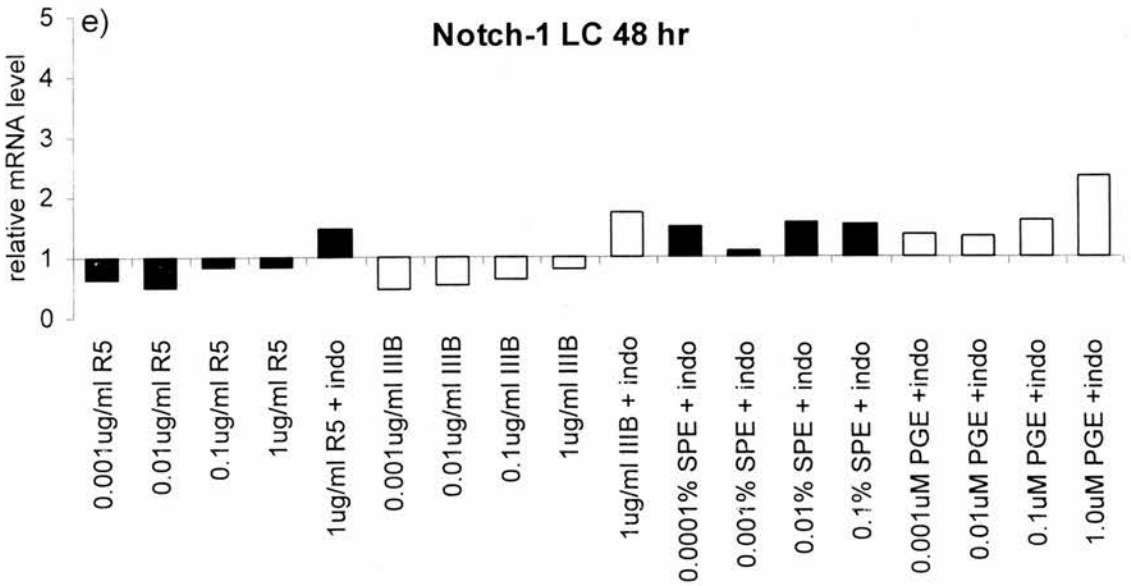

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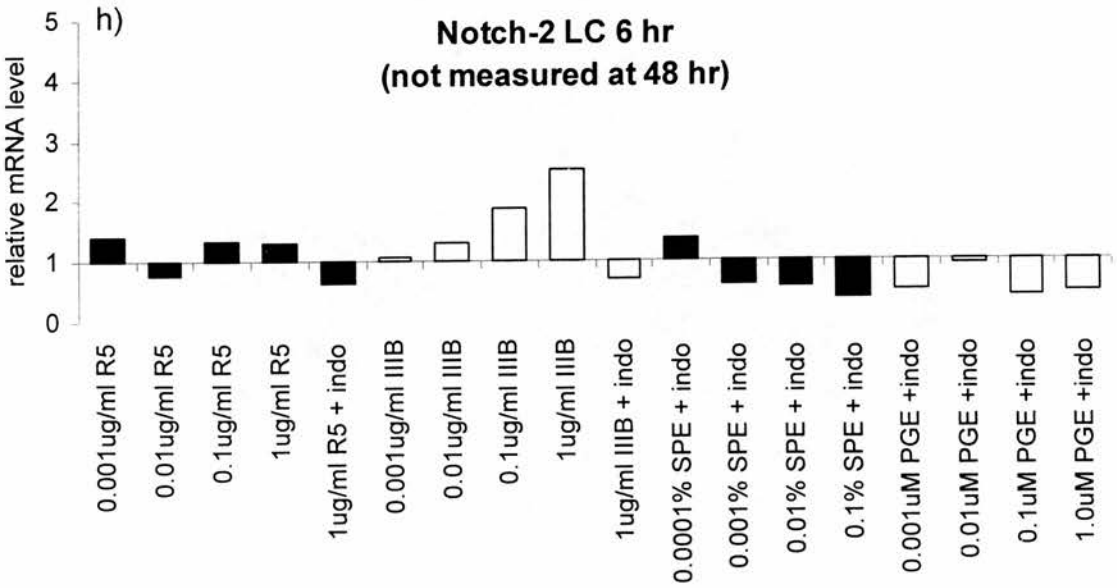

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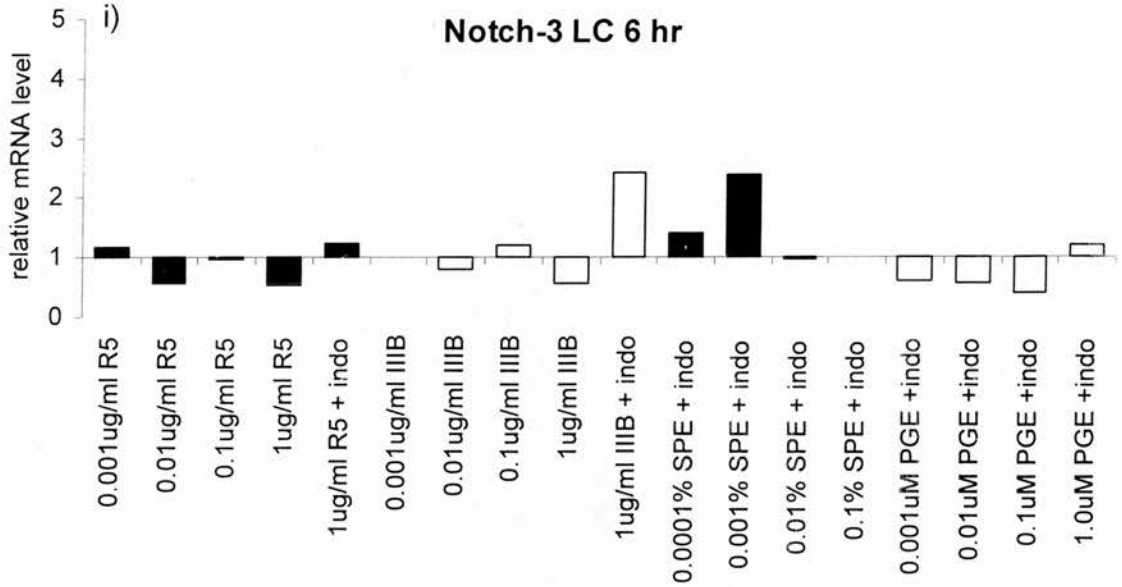

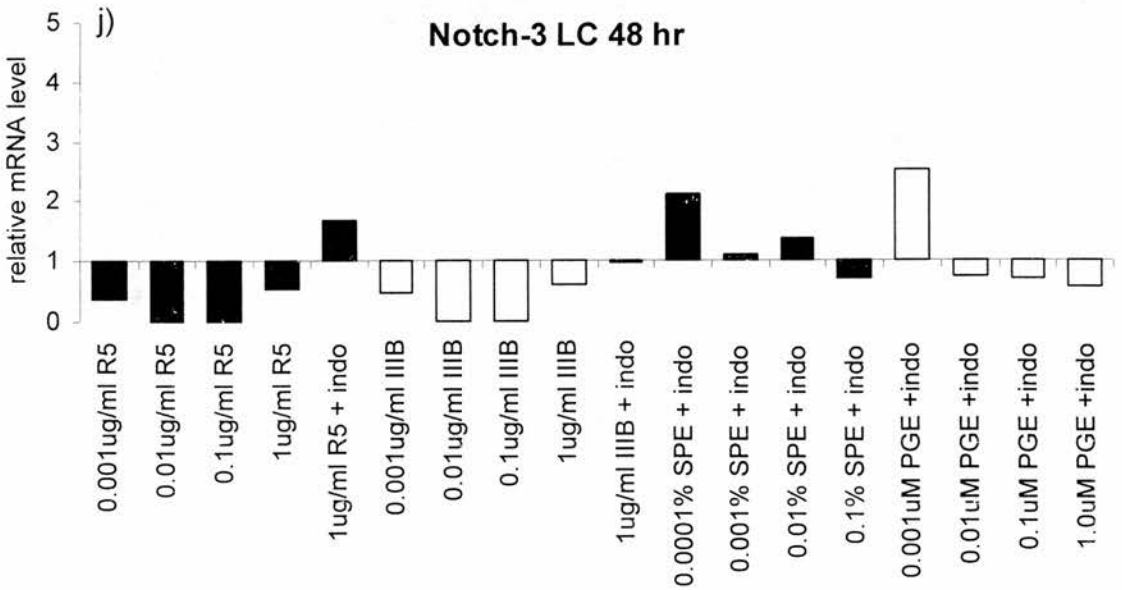

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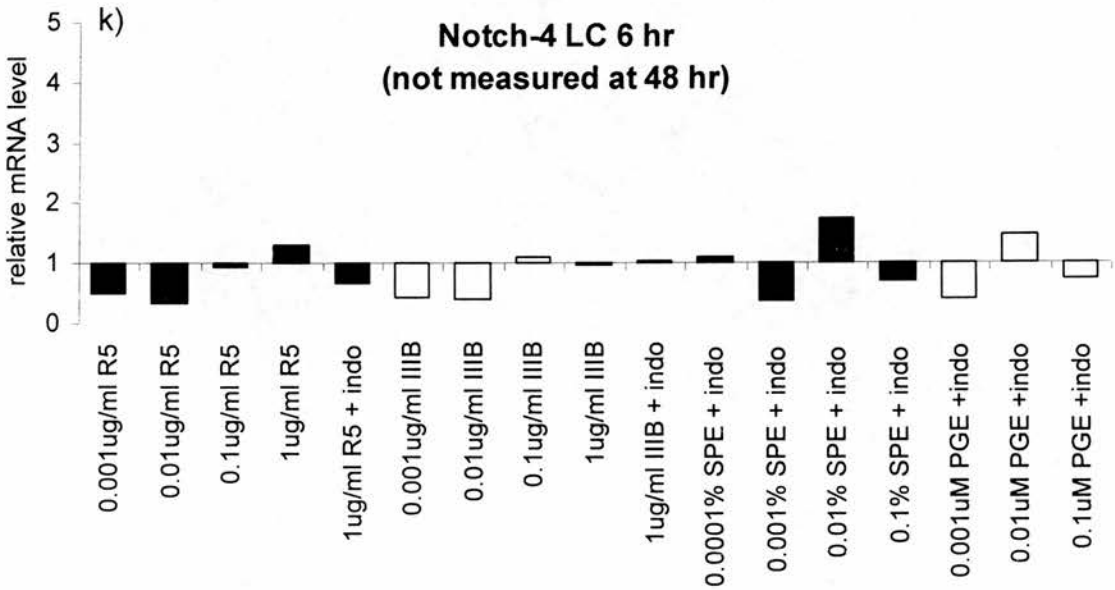

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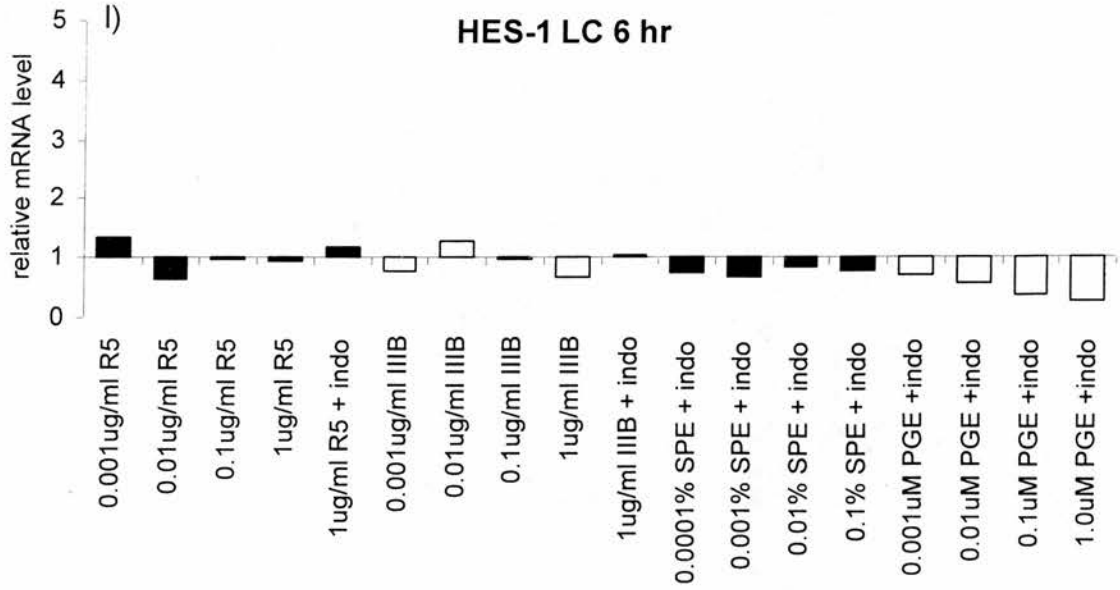

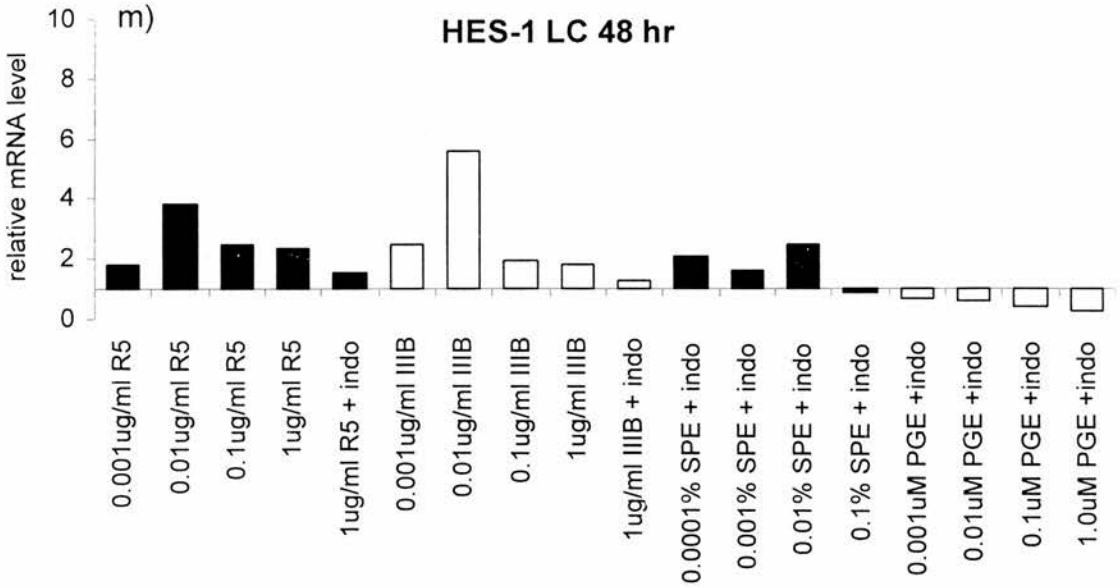

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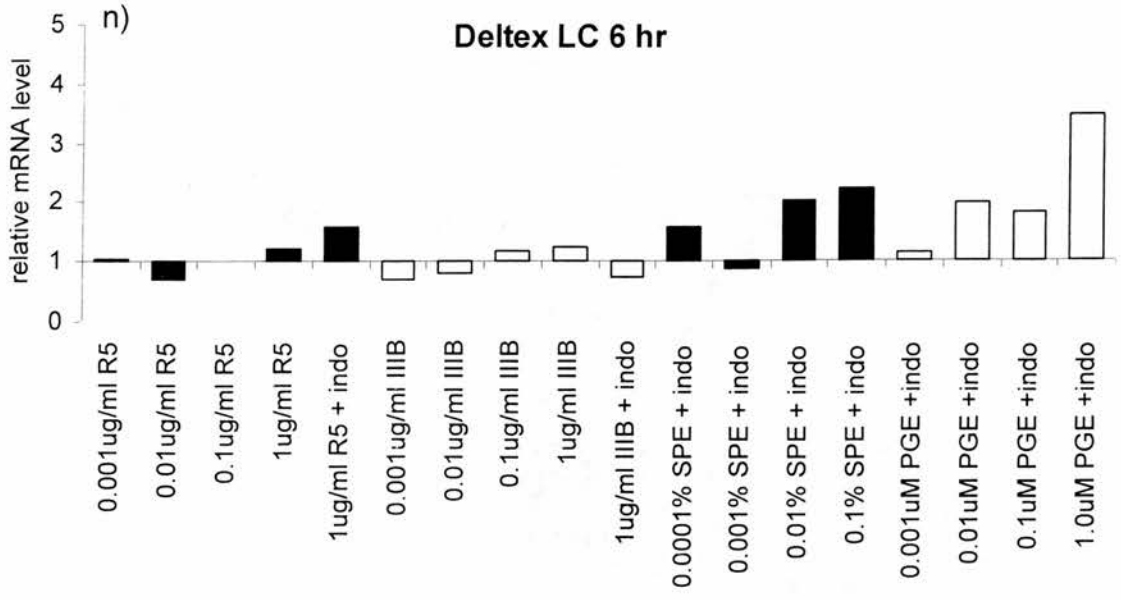
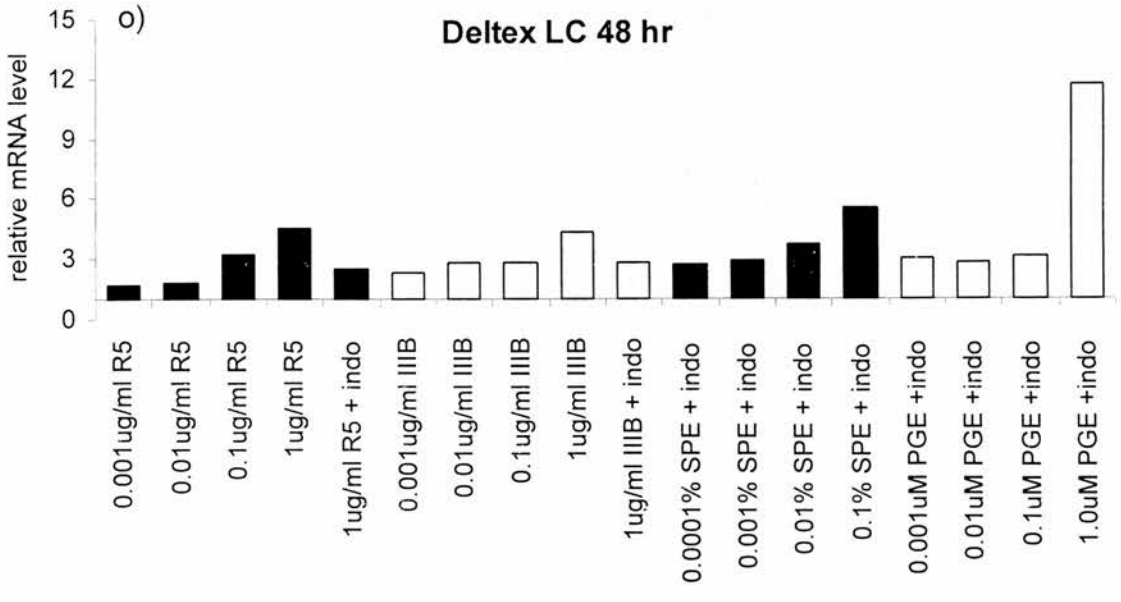




(see over for summary table and caption)

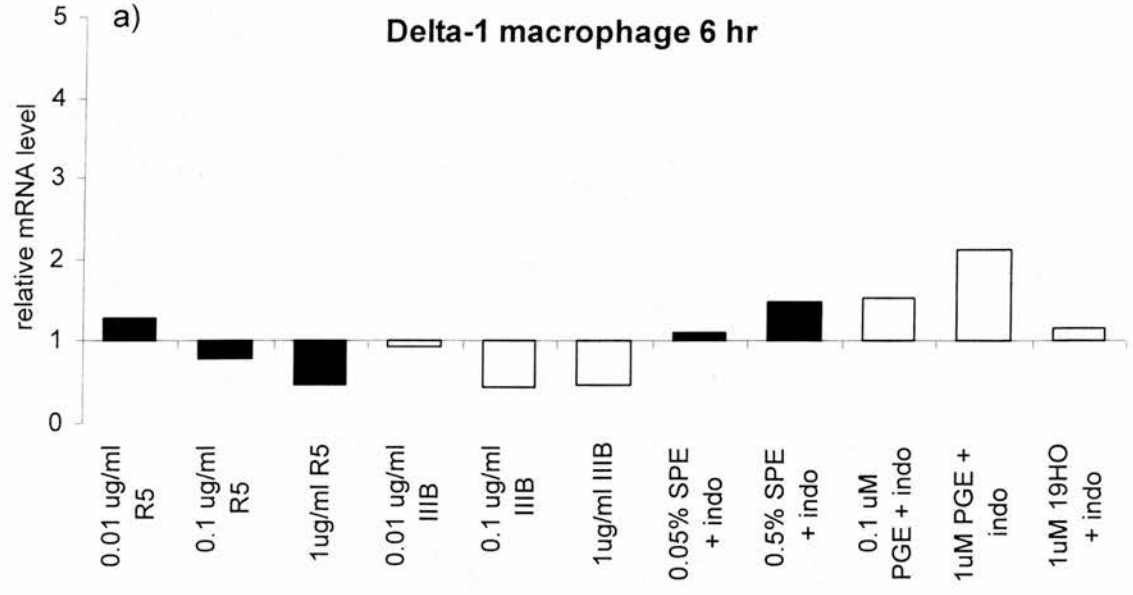
Figure 5.11 summary table

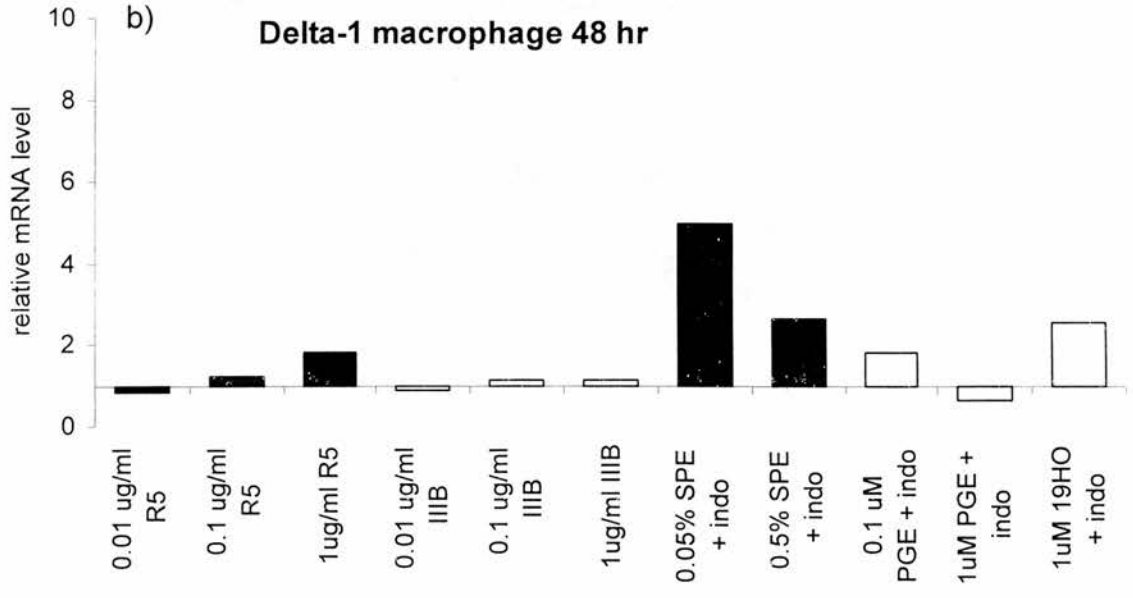
Substantial mRNA increases?	6 hours				48 hours			
	R5-gp120	gp120 IIIB	SPE	PGE	R5-gp120	gp120 IIIB	SPE	PGE
serrate-1	-	-	-	-	-	-	-	-
serrate-2	-	-	-	-	N.D.	N.D.	N.D.	N.D.
delta-1	+*	+*	+	+	-	-	-	-
notch-1	-	-	-	-	-	-	-	-
notch-2	-	-	-	-	N.D.	N.D.	N.D.	N.D.
notch-3	-	-	-	-	-	-	-	-
notch-4	-	-	-	-	N.D.	N.D.	N.D.	N.D.
HES-1	-	-	-	-	+*	+*	-	-
deltex	-	-	+	+	+*	+*	+	+

* = abrogated by indomethacin, N.D. = not determined

Only substantial specific mRNA increases observed over a range of concentrations are indicted in the above summary table. Smaller and less consistent increases have been excluded, see preceding graphs for complete unedited data.

Figure 5.11. mRNA levels in LCs. Primary LCs were treated for 6 or 48 hours with various concentrations of R5-tropic gp120, $gp120_{IIIB}$, SPE or PGE_2, in the presence or absence of indomethacin (indo). Levels of specific mRNAs, extracted from these cells, were measured by real-time PCR, and are expressed relative to basal levels in untreated cells (given a value of 1). Graphs show data from a single experiment, which was partly repeated to give similar results. Graph data is simplified and summarised in the above table





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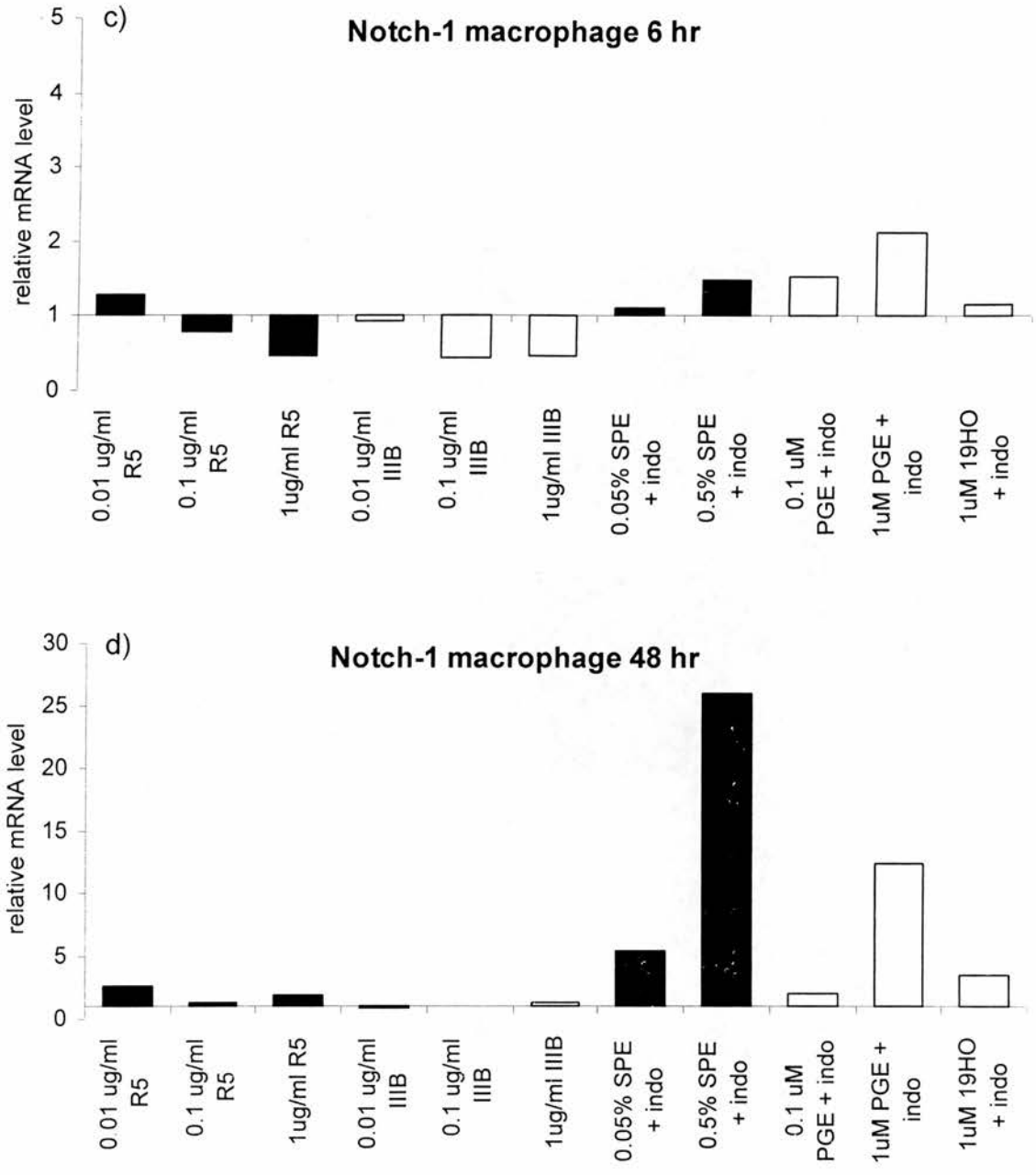
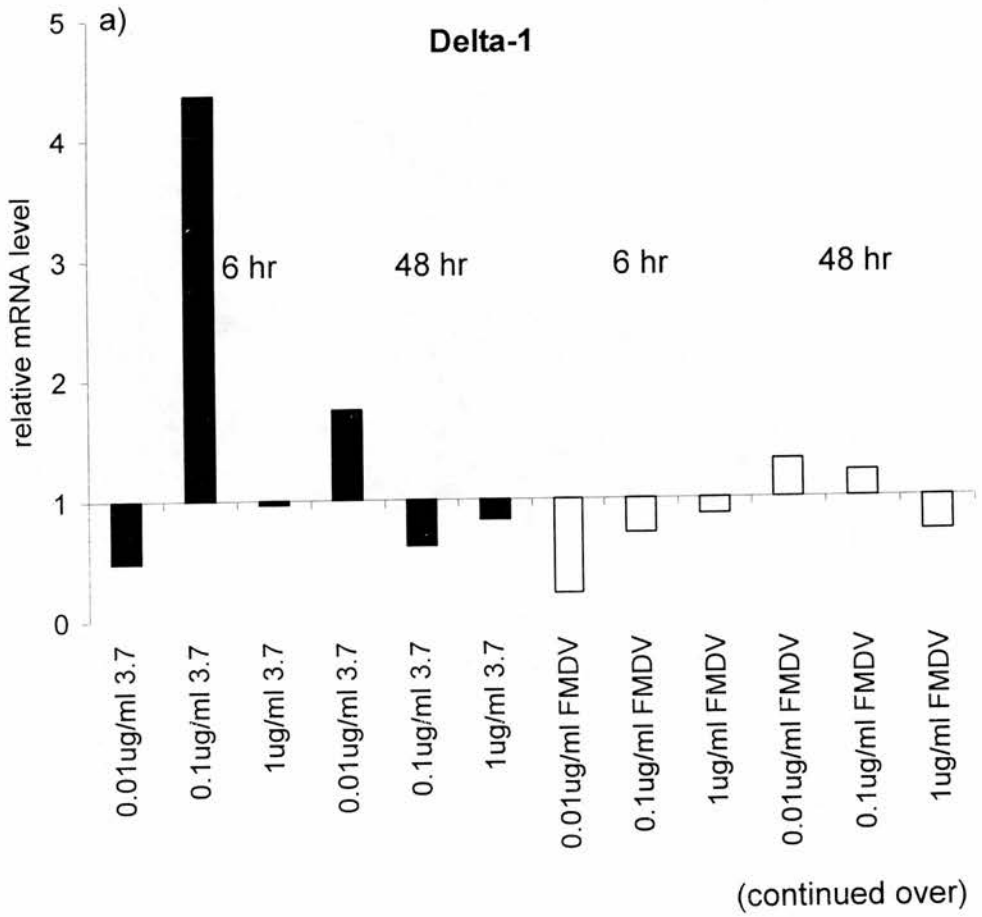


Figure 5.12. mRNA levels in macrophages. Primary macrophages were treated for 6 or 48 hours with various concentrations or R5-tropic gp120, $gp120_{IIIB}$, SPE, PGE_2 or 19HO-PGE_2, in the presence or absence of indomethacin (indo). Levels of specific mRNAs extracted from these cells were measured by real-time PCR, and are expressed relative to basal levels in un-stimulated cells (given a value of 1).



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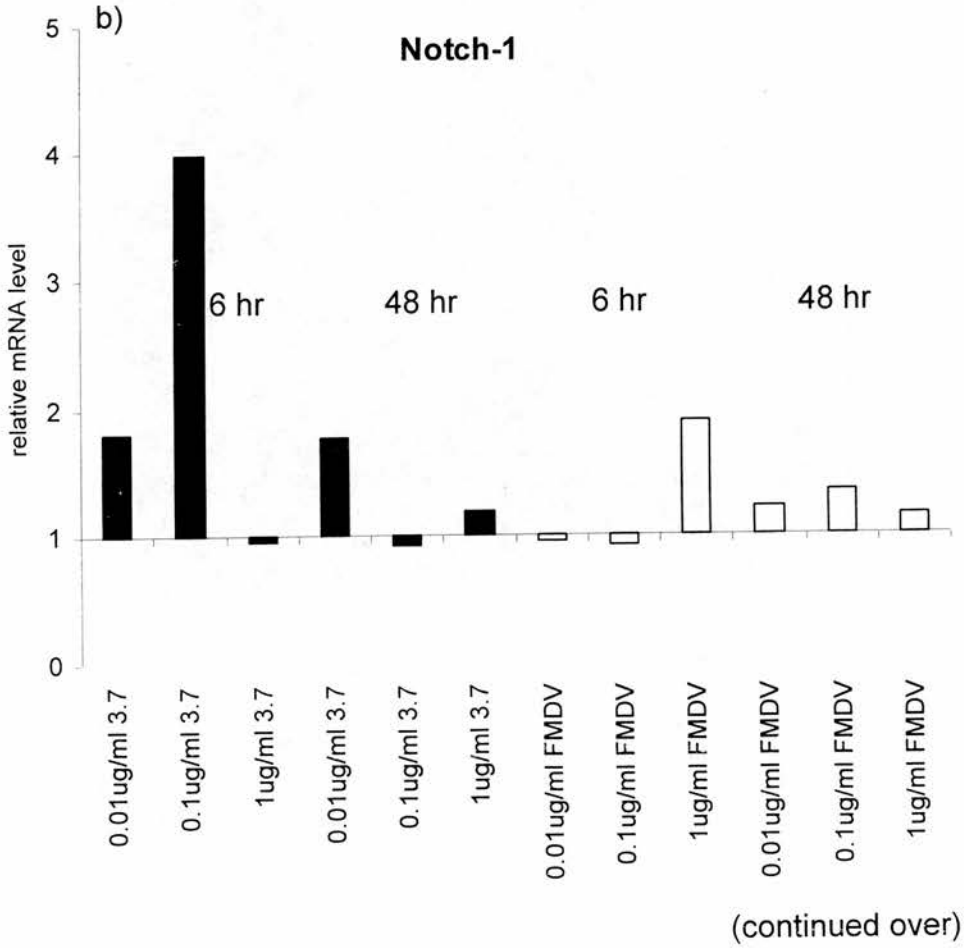

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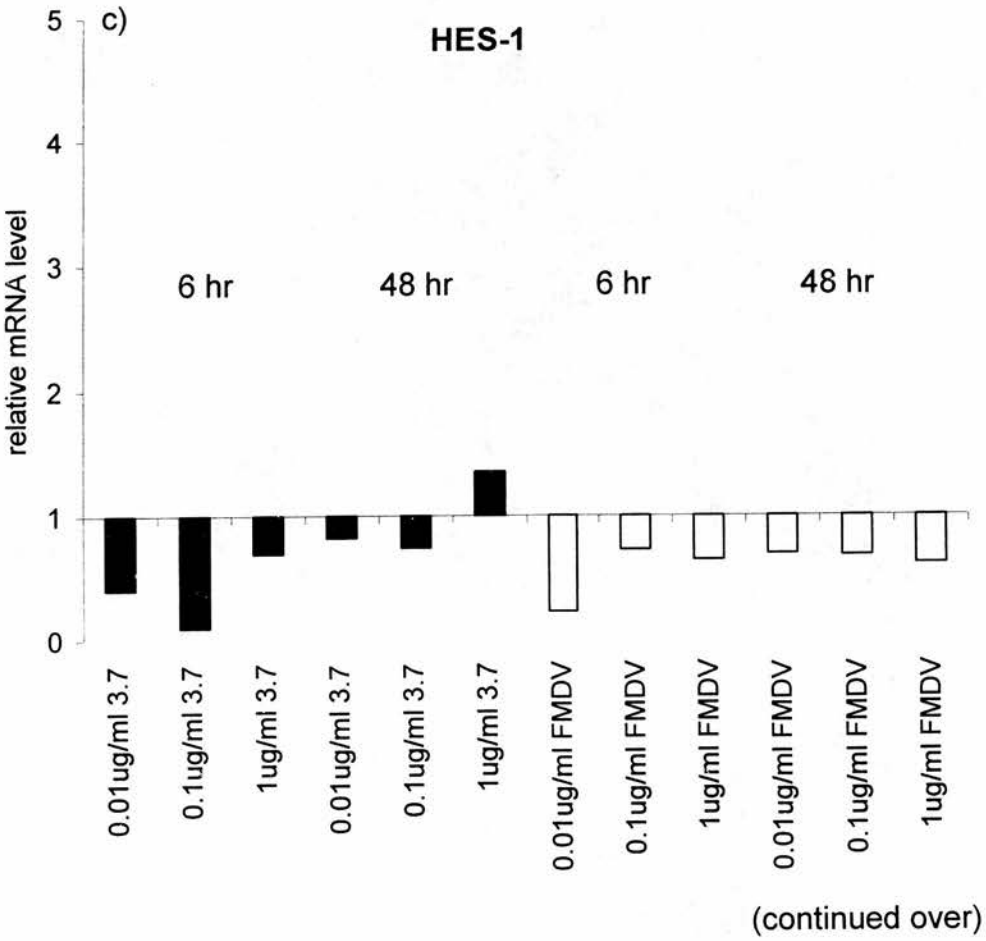

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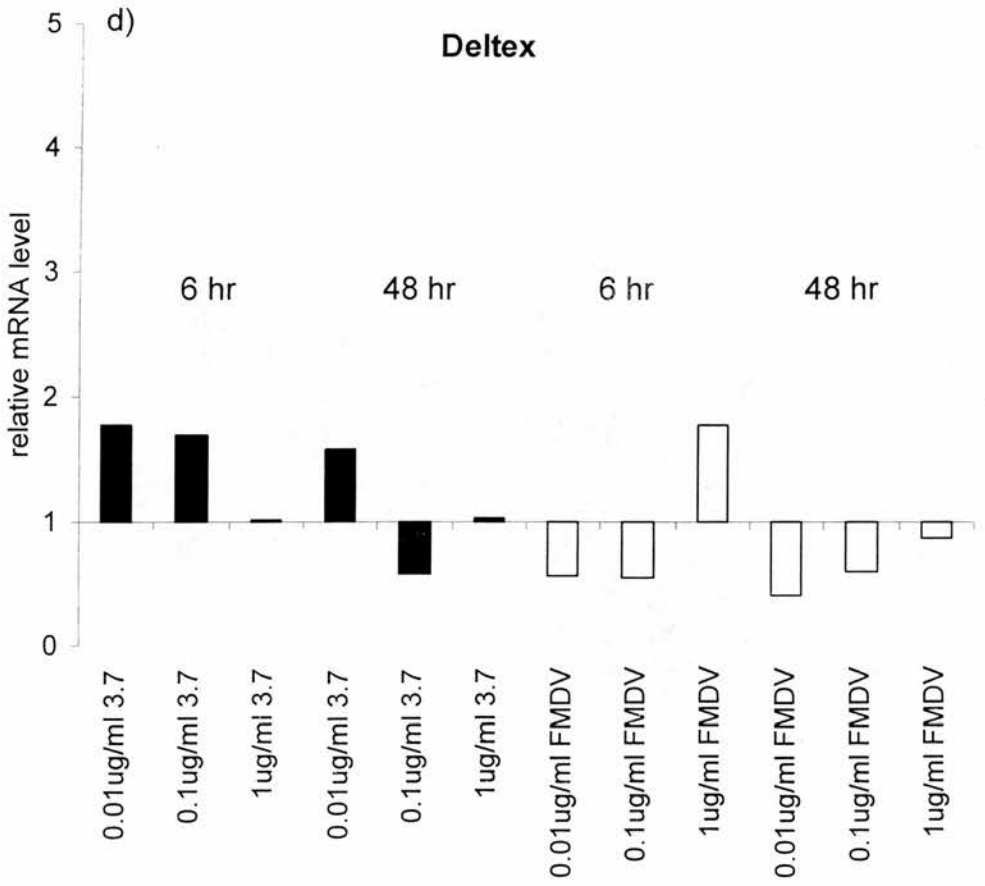


Figure 5.13. mRNA levels in LCs following peptide treatment. Primary LCs were treated for 48 hours with various concentrations of the gp120-based peptide 3.7 (black bars) or the irrelevant FMDV-based control peptide (white bars). Levels of specific mRNAs extracted from these cells were measured by real-time PCR, and are expressed relative to basal levels in untreated cells (given a value of 1).

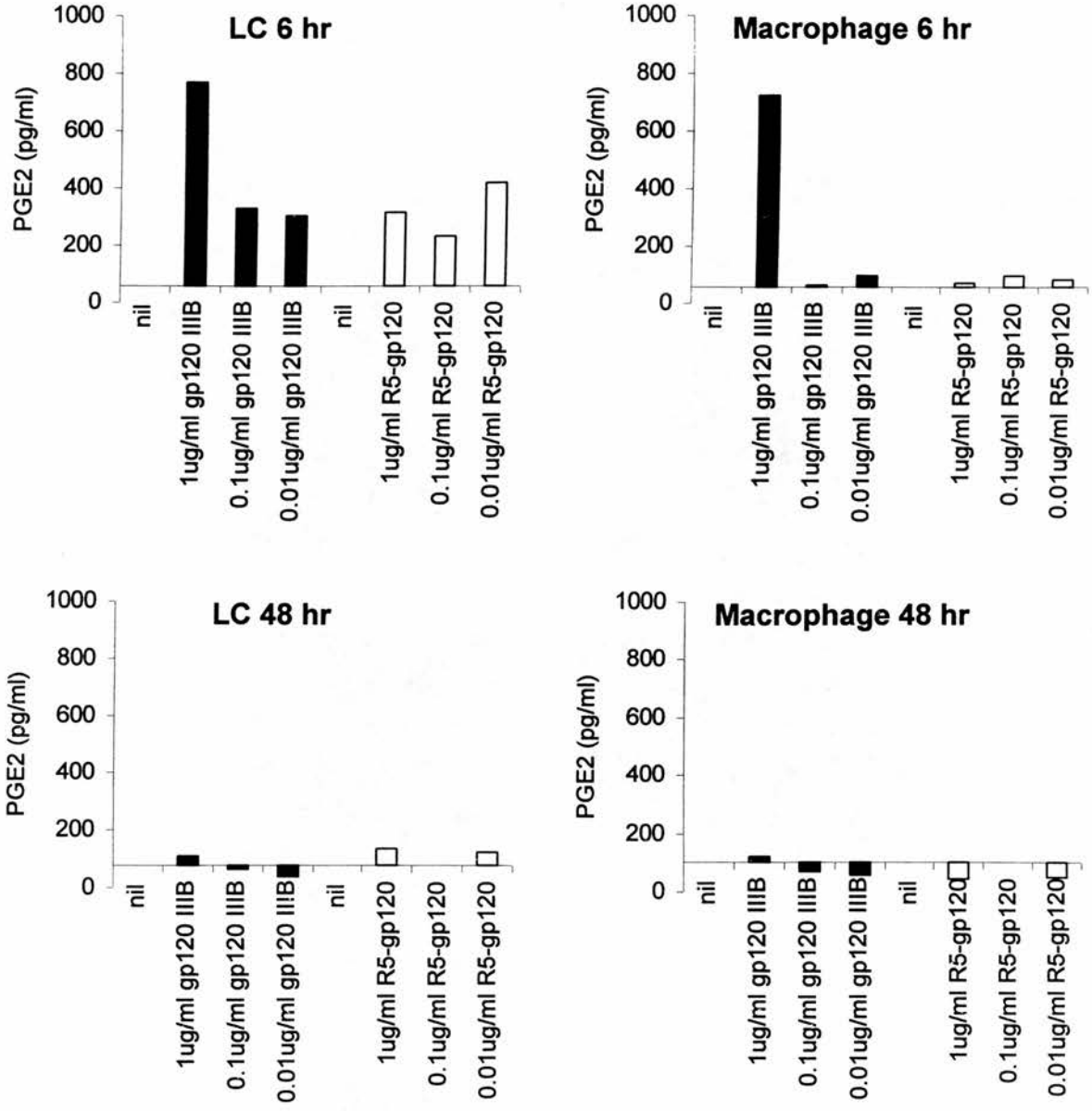
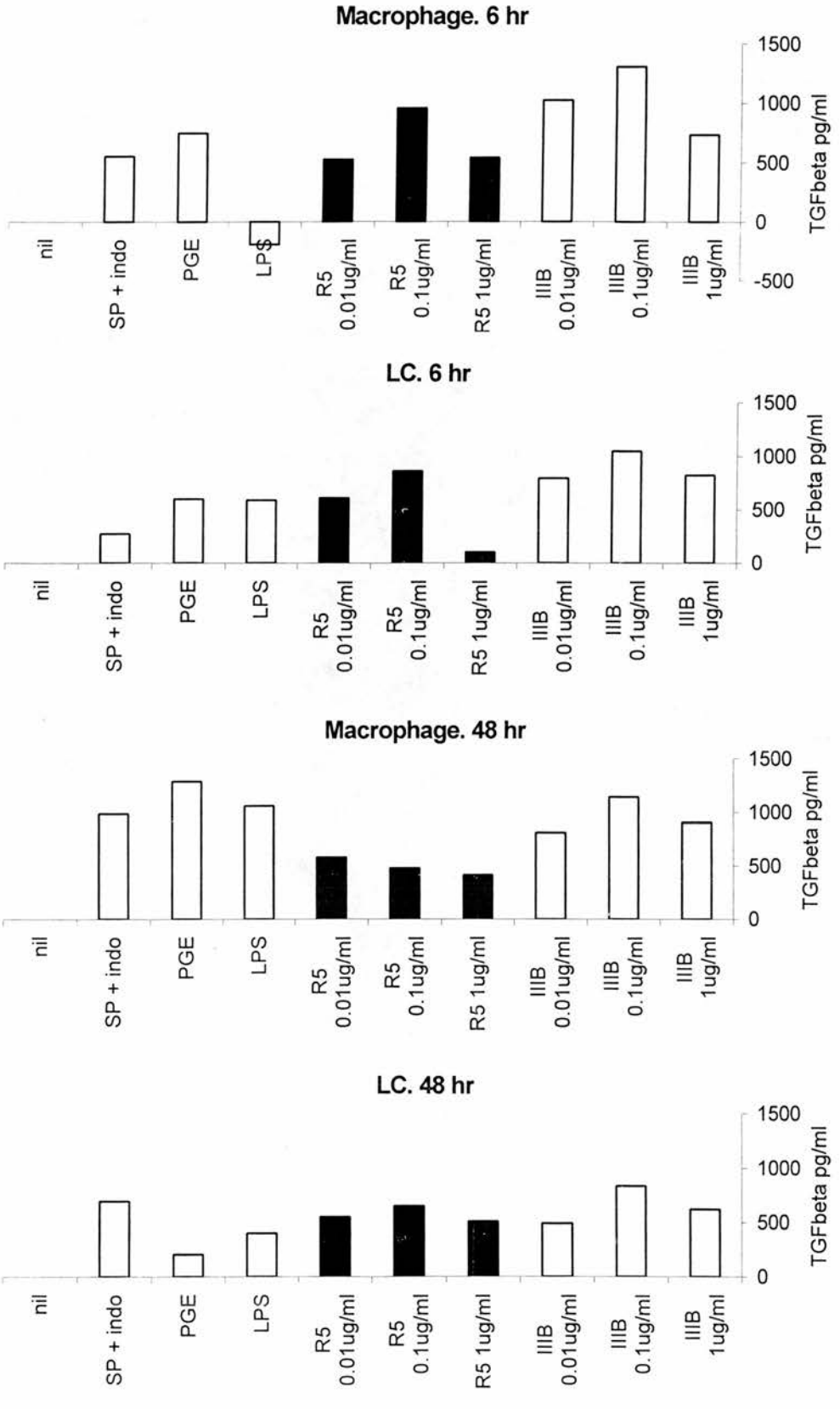


Figure 5.14. APC production of PGE_2. LCs or macrophages were treated for 6 or 48 hours with $gp120_{IIIB}$ (black bars) or R5-tropic gp120 (white bars) at the concentrations indicated. Cell culture supernatants were then assayed for PGE_2. Absolute PGE_2 concentrations are expressed as a change from that present in the supernatant or untreated cells.



(caption over page)

Figure 5.15 (previous page). APC production of TGF-β1. Macrophages or LCs were treated for 6 or 48 hours with 0.05% seminal plasma extract and indomethacin (SP + indo), 1μM PGE, 1μg/ml LPS, or various concentrations of R5-tropic gp120 or $gp120_{IIIB}$. Cell culture supernatants were then assayed for TGF-β1. TGF-β1 concentrations are expressed as changes from the concentration present in the supernatant of untreated cells.

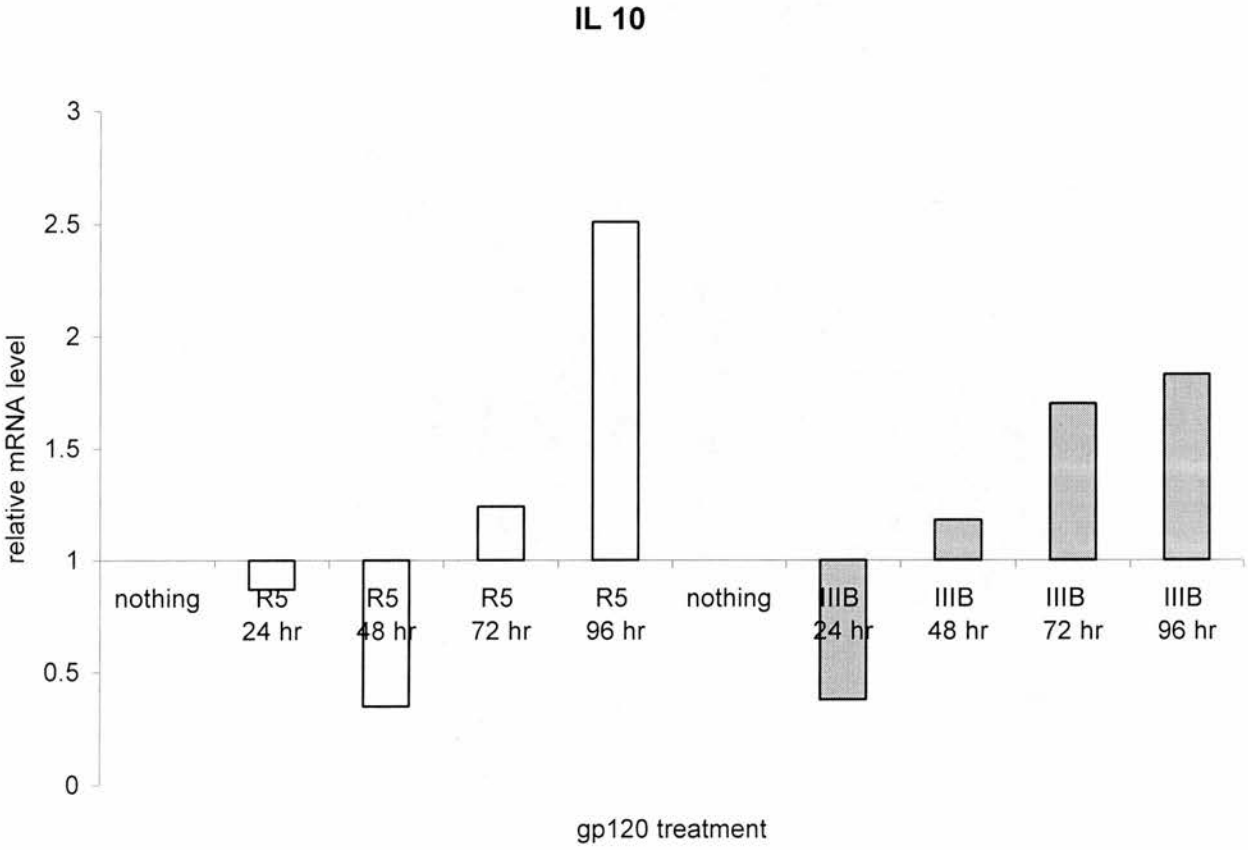


Figure 5.16. IL-10 mRNA production by macrophages. Macrophages were treated with R5-tropic gp120 (white bars) or $gp120_{IIIB}$ (shaded bars) for the times indicated. Levels of *IL-10* mRNA extracted from these cells were measured by semi-quantitative RT-PCR relative to *β-actin* mRNA, and are expressed in proportion to basal levels in untreated cells (given a value of 1).

Discussion

HIV-1 induced immune deficits = tolerance?

HIV-1 infection and HIV-1 proteins, especially gp120, can induce dysregulation of T-cells and APCs (Hewson *et al.*, 1999). Altered cytokine production (Romagnani *et al.*, 1994a; Gessani *et al.*, 1997; Bornemann *et al.*, 1997), changes in cell surface molecules (Oberg *et al.*, 1997; Stent and Crowe, 1997a; Hewson and Howie, 1998), and reduced presentation efficiency (Chelen *et al.*, 1995) by APCs can all result in a reduced lymphocyte response to both HIV-1's antigens and the antigens of other invading pathogens (Wang *et al.*, 1994; Heinkelein *et al.*, 1997; Bouhdoud *et al.*, 2000). This reduced immune response can be interpreted as the induction of inappropriate tolerance, and not just an overall immune system decline, because loss of protective responses is at least partially antigen specific (Bouhdoud *et al.*, 2000). The role of cytokines in HIV-1 induced tolerance induction has been studied for many years. The Notch signalling pathway has recently been implicated in tolerance induction in certain experimental systems (Lamb *et al.*, 1998; Hoyne *et al.*, 2000; Hoyne *et al.*, 2000), but no previous work has studied the Notch pathway in relation to immune defects induced by HIV-1 or any other infectious pathogen.

Notch and HA1.7 T-cells

T-cell lines such as HA1.7 and AC1.1 can be activated or tolerised *in vitro* by CD3/CD28 cross-linking or high dose antigen respectively (Pala *et al.*, 2000). It is not known how closely these experimental manipulations reflect physiological situations, but tolerised HA1.7 cells are both anergic and regulatory (Lamb *et al.*, 1998). Notch ligand mRNA is up-regulated when HA1.7 cells are activated or anergised (figure 5.5). However, whereas in activated cells ligand mRNA quickly returns to resting levels, anergised HA1.7 cells maintain elevated levels of *serrate-2* and especially *delta-1* mRNA for at least 120 hours. Anergic / regulatory HA1.7 cells could therefore potentially express elevated surface levels of Notch ligands indefinitely (or for as long as they remain anergic / regulatory). This suggests a mechanism by which regulatory HA1.7 cells can spread their anergic phenotype to

other HA1.7 cells, as has been previously observed (Lamb *et al.*, 1998). Resting HA1.7 cells express mRNA for all the Notch proteins and, if we assume that this mRNA is translated, they ought to be able to receive anergising signals from regulatory HA1.7 cells through these receptors. Indeed, *notch* mRNA appears to be up-regulated following anergy induction (figure 5.5) – possibly due to Notch engagement and cleavage necessitating replenishment of surface Notch protein.

However, because HA1.7 cells (in common with primary $CD4^+$ T-cells, figure 5.10) do not express *HES-1* and do not modulate *deltex* mRNA levels in response to Notch signalling, the ability of T-cells to modulate down-stream cellular events subsequent to receiving Notch-mediated signals is cast in doubt. It may be that signalling events down-stream of Notch engagement are mediated in T-cells by as yet unidentified molecules, not examined in these experiments. An alternative explanation is that although regulatory T-cells can express Notch ligand, they are unable to deliver a tolerisation signal directly to other T-cells. The spreading of an anergic phenotype from a regulatory T-cell to a naive T-cell may require to be mediated via a DC. LCs (a DC subclass) could transduce Notch signals from a regulatory T-cell via HES-1 and Deltex (figure 5.11), and then, possibly, induce anergy in naive T-cells by more traditional tolerising signals (conceivably cytokines, CTLA-4, lack of co-stimulation).

HA1.7 T-cell proliferation, gp120 and Notch

HA1.7 T-cells proliferate in response to peptide antigen presented by macrophages or LCs (figures 5.7 and 5.8). Although gp120 or anti-CD4 treatment of primary T-cells can induce *serrate-2* expression (figure 5.10), and anti-CD4 has been used to induce regulatory T-cells able to prevent graft rejection (Qin *et al.*, 1993), gp120 treatment of HA1.7 cells did not result in a reduction in the proliferative capacity of these cells (figure 5.7). Treatment of APCs with gp120, SPE or PGE_2 caused a reduction in their capacity to support HA1.7 cell proliferation (figures 5.7 and 5.8), which was not due to APC apoptosis (figure 5.9).

One explanation for the reduction in the proliferation driven by R5-tropic gp120 and anti-CD4 treated APCs could be that receptor-mediated internalisation of these proteins (as discussed in chapter 4) caused increased presentation of anti-CD4,

CD4, gp120 and CCR-5 derived peptides at the expense of $HA^{306-318}$ presentation. This mechanism would explain why $gp120_{IIIB}$ treatment of APCs did not result in a lack of proliferation, but would not explain the actions of PGE_2 and SPE.

The observation that gp120 induces prostaglandin production by LCs (figure 5.14), and that indomethacin abrogates the effects of R5-tropic gp120 and anti-CD4 on HA1.7 proliferation (figure 5.8) suggests that the gp120 and anti-CD4 induced reduction in HA1.7 proliferation may be the result of immunosuppressive prostaglandin release. However, this cannot be the whole story because prostaglandin is released in response to gp120 in a non-strain specific fashion (figure 5.14), and other immunosuppressive cytokines including TGF-β1 (figure 5.15) are present.

Gp120 induces the expression of Notch ligand mRNA, especially that for Serrate-2 (figure 5.11). It is possible, therefore, that the Notch pathway is involved in the inhibition of HA1.7 proliferation, although this explanation on its own it unable to account for the lack of strain specificity in gp120 induction of Notch pathway molecules. Because prostaglandin treatment of LCs inhibits HA1.7 proliferation (figure 5.8), and gp120 induces prostaglandin release in a non-strain specific manner (figure 5.14), it is likely that gp120 is immunosuppressive because it stimulates prostaglandin release, which in turn stimulates the expression of Notch ligands. Expression of Notch ligand mRNA is partly abrogated by the presence of indomethacin. Neither prostaglandin release (figure 5.14), nor *delta-1* mRNA up-regulation (figure 5.12) in response to gp120 was seen on macrophages. There is no direct evidence presented here that prostaglandin-induced expression of Notch ligands on APCs induces an anergic / regulatory phenotype in the HA1.7 cells (as opposed to just a reduction in proliferation), although transfection of APCs to express Notch ligand is known to do this (Lamb *et al.*, 1998).

As well as expressing Notch ligand in response to tolerising agents, LCs express mRNA for Notch proteins (figure 5.11) and therefore ought to be able to receive, as well as give, Notch-mediated signals. *HES-1* (figure 5.11m) and *deltex* (figure 5.11o) mRNA is up-regulated in LCs in response to gp120, SPE and PGE_2. This is evidence for these cells receiving Notch signals, something macrophages may be unable to do due to the absence of *HES-1* or *deltex* mRNA. The timing of the mRNA expression of members of the Notch pathway in LCs is interesting. Notch

ligand mRNA is up-regulated from 6 hours, but changes in *HES-1* and *deltex* levels are first measured at 48 hours. The delay in *HES-1* and *deltex* expression may represent the time taken for intervening signalling steps.

Viewed as a whole, the data in this thesis do not point to a single mechanism of gp120-induced immunosuppression, which is able to account for the strain specificity of gp120-induced proliferation defects, gp120-induced reduction in the proliferation-supporting capacity of both macrophages and DCs, and the effects of indomethacin. It therefore seems likely that presentation efficiency, prostaglandin, Notch ligands, and possibly TGF-β1 are all involved in gp120 induced immune dysregulation.

Semen and Notch

Semen is profoundly immunosuppressive, largely due to its high prostaglandin concentration (Kelly, 1999). SPE and PGE_2 induced the production of TGF-β1 by APCs (figure 5.15). An additional mechanism of immunosuppression by semen may be the induction of APCs to express Notch ligands (figures 5.11 and 5.12). The induction of Notch ligands by SPE and PGE_2 has important implications for the initiation of protective immune responses against sexually transmitted pathogens (including HIV-1) delivered in the immunosuppressive seminal environment (Kelly, 1997; Kelly and Critchley, 1997b).

DCs are special

HA1.7 cells, primary $CD4^+$ T-cells, macrophages and LCs all express Notch ligand mRNA and presumably Notch ligand surface protein. However only LCs express the complete machinery (*HES-1* and *deltex*) for transduction of an incoming Notch signal along all known intracellular pathways. Macrophages express neither *HES-1* nor *deltex*, and T-cells express *deltex* only but do not appear to modulate its mRNA level upon reception of a Notch signal (at least not in the studies presented here). The difference between macrophages and LCs in this regard is especially startling when it is remembered that the LCs and macrophages used in the experiments presented here were both differentiated from the same population of peripheral blood monocytes. It

would be interesting to correlate the acquisition of dendritic/Langerhans surface markers with *deltex* and *HES-1* mRNA expression during LC differentiation. The discovery that only LCs (and presumably other DCs) express *HES-1* and *deltex* may point to a unique role of DCs in immune regulation. All immune system cell types can potentially receive tolerisation signals from pathogens or cytokines and change their own phenotype accordingly (this would include up-regulation of Notch ligands). However, if DCs are the only cell type able to receive Notch mediated signals in a complete form able to cause the up-regulation of their own Notch ligands, DCs will be required for the reception of Notch signals from regulatory T-cells. If regulatory T-cells principally regulate the activity of other lymphocytes via the expression of Notch ligands, it follows that DCs may be required for the activity of regulatory T-cells. T-cell to T-cell tolerance inducing signals would have to be mediated via a DC (figure 5.1). Such a requirement may have evolved as a way of strictly controlling the antigen specificity of tolerance induction, and limiting the spread of epitope specificity during linked and bystander suppression to those epitopes presented by the same APC. However, under some circumstances T-cells can be rendered anergic by Notch ligand expression on APCs (Lamb *et al.*, 1998), so they must be able to transduce signals from Notch without the help of HES-1, possibly solely by the use of deltex, although in the studies presented here T-cell *deltex* mRNA levels never changed.

CHAPTER 6: RESULTS

ANTI-HIV-1 PEPTIDES

Background

Anti-HIV-1 drugs

HIV-1 is the most sought after drug target ever, but after almost 20 years of effort there is still no cure for AIDS. Academic and commercial research groups have found many interesting molecules, from diverse sources, which have exhibited anti-HIV-1 properties *in vitro*. However, only 14 drugs (in just three classes) have been licensed (in the UK, Parfitt, 1999) for treatment of HIV-1 infection. None of these drugs is satisfactory due to toxicity problems, the rapid evolution of resistance and the failure to produce a complete and permanent cure (Moyle, 2000).

Current anti-HIV-1 drugs

The replicative cycle of HIV-1 can be split into 10 stages (Mohan and Baba, 1995), *adsorption* (initial virion binding to the cell surface), *fusion* of the envelope and plasmalemma (these first two stages comprise *entry*), *uncoating* of the viral genome, *reverse transcription*, *integration* of provirus into the host genome, *DNA replication*, *transcription* of viral mRNA, *translation* of viral genes, *maturation* of viral proteins and *budding* (assembly and release of virus from the cell surface). A drug could potentially block any stage of the replicative cycle. There may be advantages in treating HIV-1-infected individuals with a combination of drugs; each targeted to a different stage in the viral replicative cycle, to reduce the likelihood of viral resistance to the complete treatment protocol from developing. Drugs currently in use include reverse-transcriptase inhibitors such as AZT and protease inhibitors such as Indinvar, which target the viral protease needed to insure correct *maturation* of viral proteins. Targeting the *fusion* stage with peptides and plant lectins (Matsui *et al.*, 1990; Balzarini *et al.*, 1992), the *uncoating* stage with bicyclams (DeClercq *et*

al., 1992), and the *integration, DNA replication, transcription* and *translation* stages with antisense oligonucleotides (Zamecnik *et al.*, 1986; Sarin *et al.*, 1988; Goodchild *et al.*, 1988; McShan *et al.*, 1992) has also been attempted but has not yet resulted in widely-used clinical drugs.

HIV-1 entry inhibitors

Within the past year a dendritic cell (DC) specific protein, DC-SIGN (Geijtenbeek *et al.*, 1999; Steinman, 2000; Geijtenbeek *et al.*, 2000b) has been identified as an HIV-1 receptor (Geijtenbeek *et al.*, 2000a) which mediates the very first interactions between HIV-1 and the DC. There have been suggestions that disrupting this interaction might be an effective drug target. No work has yet been published on DC-SIGN inhibitors but they may be promising because the DC-SIGN interaction is of high affinity and its expression is restricted to DCs. However, DC-SIGN-mediated interactions with T-cells are important in supporting primary immune responses (Geijtenbeek *et al.*, 2000b) so there is a danger that a therapy aimed at blocking the function of DC-SIGN could cause an immune deficit (extremely undesirable in an AIDS patient) as a side effect.

A more promising and better-studied target for intervention is the gp120 / gp41 / CD4 / chemokine receptor interaction. Targeting this early step in the viral replication cycle is attractive because it will prevent the spread of virus before cellular entry thereby allowing HIV-1 the least opportunity to do harm. Because binding of HIV-1 to cells involves host proteins that do not mutate during the course of infection and corresponding viral proteins that have their variability constrained at key residues by their need to bind to these host proteins, there may be less of a problem with viral resistance to drugs targeted to the entry stage of HIV-1's replication cycle.

Even if absorption or entry inhibitors fail to function successfully as a treatment for established HIV-1 infection, they may have a role as 'morning after', post exposure drugs aimed at stopping the establishment of a new infection. Because such drugs ought to require just a few doses given under medical supervision, it would not be necessary to deliver them orally; this might overcome problems with the oral bioavailability of some drugs candidates (especially protein or peptide based

treatments). The World Health Organization has identified an urgent need for tropically applied HIV entry inhibitors suitable for use by women to block sexual transmission of HIV-1 (Lange *et al.*, 1993; The International Working Group on Vaginal Microbicides, 1996; Elias and Coggins, 1996). Entry inhibitors that fall short of curing HIV-1 infection might prolong life and increase its quality by reducing the rate of cellular dissemination of HIV-1 and by interfering with the CD4 / chemokine receptor / gp120 / gp41 interactions involved in syncytium formation (Levy, 1994). A reduction of the syncytia inducing ability of HIV-1 would be beneficial to the patient by reducing the opportunity for viral recombination (Burke, 1997), decreasing HIV-1 replication dynamics (Connor and Ho, 1994; GranelliPiperno *et al.*, 1995) and slowing the loss of immune cells. Because most (but not all) syncytium inducing (SI) HIV-1 strains use CXCR-4 as a coreceptor (Zhang *et al.*, 1998; Horuk, 1999; Abebe *et al.*, 1999), drugs targeted to inhibit syncytium formation would be better targeted to block CXCR-4 than CCR-5.

Absorption inhibitors have five main targets, CD4, gp120, gp41 and the CXCR-4 or CCR-5 coreceptor. Targeting an inhibitor to a host protein might avoid the problem of resistance evolving. However, a drug that binds to host proteins might produce unwanted side effects by disrupting the physiological roles of these proteins as receptors for MHC class II, IL-16 and chemokines respectively. Studies of the *ccr5Δ32* mutation (see chapter 3) show that loss of CCR-5 function is not harmful (Berger, 1997); this suggests that drugs designed to specifically block CCR-5 would be well tolerated. CCR-5 might be a better target than CXCR-4 (particularly for a drug to be given shortly after exposure). Protecting APCs from infection might be more important than protecting T-cells because APCs have a longer life span and less capacity for renewal than T-cells, and because APCs are the first cells to be infected after virus transmission (Miller *et al.*, 1989; Spira *et al.*, 1996). One side effect of the use of CCR-5 inhibitors might be the selection for new and more pathogenic HIV-1 strains using CXCR-4 (Michael and Moore, 1999) or possibly another, currently rarely used, chemokine receptor (Landau, 1997).

Current HIV-1 entry inhibitors

Several different groups of chemical entities have been demonstrated to be HIV-1 entry inhibitors. These potential drugs have arisen from the screening of natural and synthetic chemicals, variations of the proteins involved in HIV-1 absorption (chemokine receptors, CD4 and gp120) and their natural ligands (chemokines such as MIP-1α) and from rational design based on knowledge of the molecular interactions. This last approach has been made easier by the crystallographic elucidation of the structure of gp120 (Wyatt and Sodroski, 1998b) and gp41 (Chan *et al.*, 1997). There is evidence that some HIV-1 exposed but uninfected individuals may gain protection by their ability to produce high levels of CC chemokines (Paxton *et al.*, 1996; Zagury *et al.*, 1998), these observations support the strategy of using chemokine-based molecules to block infection.

One of the best-described natural entry inhibitors is cyanovirin-N, an 11 kD protein originally extracted from the cyanobacterium *Nostoc ellipsosporum* but since produced by recombinant DNA expression techniques (Boyd *et al.*, 1997; Gustafson *et al.*, 1997; Mori *et al.*, 1997a; Mori *et al.*, 1997b; Mariner *et al.*, 1998; Bewley *et al.*, 1998; Mori *et al.*, 1998; Yang *et al.*, 1999; Esser *et al.*, 1999). It appears that cyanovirin-N acts by disrupting post-absorption fusion events. Other entry inhibitors include the bis-azo compound FP-21399, which binds to gp120 (Zhang *et al.*, 1998), and a soluble galactosylceramide analogue (Fantini *et al.*, 1997). However, most entry inhibitors so far designed have been proteins, or peptides, based on one of the viral or host proteins involved in HIV-1 entry. There has been some success with peptides targeted to interfere with the conformational changes required for the fusogenic portion of gp41 to interact with the target cell membrane (Jiang *et al.*, 1993; Kilby *et al.*, 1998; Nisole *et al.*, 1999), most notably with the peptide T-20 which has entered clinical trials (Rizzardi and Pantaleo, 1999). For a time, soluble (s) CD4 and derivatives such as truncated CD4 molecules and CD4-immunoglobulin fusion proteins were promising drug candidates. These proteins were demonstrated to block infection of T-cell lines *in vitro* (Capon *et al.*, 1989; ShapiraNahor *et al.*, 1990; Rausch *et al.*, 1992; Yeh *et al.*, 1992; Meshcheryakova *et al.*, 1993) and prevent the infection of chimpanzees by the T-cell adapted HIV-1 strain IIIB (Ward *et al.*, 1991). However, when tested on macrophages, M-tropic HIV-1 strains and primary virus

isolates, sCD4-based therapies were much less effective (Daar *et al.*, 1990; Gomatos *et al.*, 1990). The misleadingly encouraging results from the T-cell adapted HIV-1 experiments might have been because these HIV-1 strains require an especially strong (and therefore more prone to disruption) interaction with cellular CD4. The sCD4 experience demonstrates how important it is to choose an appropriate assay system for *in vitro* drug screening. Most recent attempts at protein / peptide inhibitors have focused on gp120 mimics. Gp120 may be a better target than a cellular protein because HIV-1 is unlikely to be able to evolve a reduced requirement for gp120 use in the same way that a reduced requirement for CD4 or chemokine receptor binding could evolve. Many short peptides (Wang, 1989; Nehete *et al.*, 1993; Mabrouk *et al.*, 1995; Delézay *et al.*, 1996; Murakami *et al.*, 1997; Donzella *et al.*, 1998; Barbouche *et al.*, 1998; Ferrer and Harrison, 1999) and several branched peptides (Fantini *et al.*, 1993; Yahi *et al.*, 1994; Benjouad *et al.*, 1994; Yahi *et al.*, 1995; Benjouad *et al.*, 1995; Sabatier *et al.*, 1995; Fantini *et al.*, 1996) have been shown to inhibit HIV-1 infection *in vitro*. Most of these peptides incorporate either CD4 binding domains or the V3 loop, although all of them are based on either consensus sequences from North American and European HIV-1 isolates or from T-cell tropic viral sequences. Most synthetic gp120 peptides so far investigated have contained continuous gp120 sequences.

The use of gp120 / gp41-binding peptides in therapy must be approached with caution, both soluble CD4 and certain peptides derived from the V3 loop of gp120 have been shown to enhance HIV-1 infection under certain *in vitro* circumstances (Demaria and Bushkin, 1996; Dettin *et al.*, 1998), presumably by mediating conformational changes in viral gp120 or host receptor which allow for more efficient binding. Other possible side effects might be caused by artificial peptides mimicking the toxic or immunoregulatory properties of gp120 / gp41 (Werner *et al.*, 1991; Garry and Koch, 1992; Miller *et al.*, 1993).

Manufacture of peptides

Traditionally, therapeutic peptides and proteins such as insulin (Keefer *et al.*, 1981) have been made by using recombinant DNA to engineer the expression of large amounts of product in microbial, insect or mammalian cell culture. Therapeutic

peptides have also been expressed in the milk of farm animals (Colman, 1999). These genetic engineering approaches which have also been used to produce peptides for vaccination have the advantage of production of large amounts of peptide at relatively low cost. They may also allow for protein glycosylation which may (or may not) be identical to the naturally produced protein. Branched peptides cannot be made by conventional genetic engineering. Modified or non-protein amino acids and other modifications needed to increase the bioavailability or stability of the peptide cannot be made by genetic engineering. Branched peptides have the advantage of recreating more effectively the three-dimensional structure of the whole protein on which they are modelled. Three-dimensional structure may be important for binding function and for antigenicity, especially in the case of B-cell epitopes, which are often discontinuous (Janeway and Travers, 1996).

F-moc chemistry in automated peptide synthesis

Several chemical methods are available to produce completely synthetic peptides. The F-moc (9-fluorenylmethoxycarbonyl) automated synthesis technique depends on adding 1-hydroxybenzotriazole-activated esters of the required amino acids one at a time to a growing peptide chain, which is immobilised to a solid-phase 4-benzyloxybenzyl alcohol resin. The amino terminals (and any amino side groups) of the amino acids to be added are protected by F-moc, so the only available amino groups are on the immobilised peptide. Such a method allows efficient synthesis from the C-terminal to the N-terminal of peptides up to several dozen residues long (Ramage *et al.*, 1994). Branch points can be added to the linear chain by stopping the automated synthesis and adding a protected lysine, arginine or histidine ester by hand (Bycroft *et al.*, 1993; Howie *et al.*, 1998). Selective protection of the α-amino group on this residue at the end of the nascent peptide allows automated synthesis to be restarted from a side-chain amino group along one of the peptide branches. The protection can then be removed from the α-amino group and automated synthesis down the second branch started. F-moc chemistry is not limited to the synthesis of peptides solely from the amino acids used in nature.

Anti-HIV vaccines

Although treatment for HIV-1 infected people is an important goal, a successful treatment is likely to be unaffordable in the Developing World, where most HIV-1 infections and deaths occur. Although simple, preventative measures such as the promotion of safer-sex which has recently yielded very encouraging results in Thailand (Phoolcharoen, 1998) are needed, a cheap and effective AIDS vaccine is the only realistic hope that most of the Third World has of solving its HIV problem. The vaccine approach may be more effective in preventing infection than anti-retroviral therapy ever is at eradicating infection because of HIV-1's ability to hide as a provirus in the host genome, invisible to the immune system and drug treatments (McCune, 1995). It has recently been estimated that the half-life of this latent viral reservoir is about 44 months (Finzi *et al.*, 1999; Persaud *et al.*, 2000); this would mean that even a small reservoir of 1×10^5 cells would take at least 60 years to clear (Gotch and Hardy, 2000) unless virus were forced out of hiding by immunotherapy such as IL-2 administration (Davey *et al.*, 1999). Alternatively, therapeutic vaccines may be useful adjuncts to existing anti-viral therapies (Gotch *et al.*, 1999).

The immune system is strongly activated against HIV-1 in infected people. The emergence of immune-escape HIV-1 variants (Goulder *et al.*, 1997; Harcourt *et al.*, 1998) is evidence that the immune system places HIV-1 under selective pressure at least as great as that from anti-retroviral drugs (Borrow *et al.*, 1997; Price *et al.*, 1997; Gotch and Hardy, 2000). B-cell immunity to HIV-1 is seen in infected people; this can control HIV-1 by blocking infection, opsonisation or by complement-mediated lysis (Gotch and Hardy, 2000). $CD8^+$ CTL and $CD4^+$ Th-cell responses are also seen in HIV-1 infection (Berzofsky, 1991). As well as causing lysis of infected cells, T-cells can produce CCR-5-blocking chemokines such as MIP-1α and RANTES (Park *et al.*, 1999; Polo *et al.*, 1999) and the distinct $CD8^+$ T-cell antiviral factor (Hsueh *et al.*, 1994; Mackewicz *et al.*, 1994; Levy *et al.*, 1996). There are several unknowns as to which responses offer significant protection from disease and ought to be strengthened by prophylactic vaccination. Anti-gp120 IgG antibodies can block infection *in vitro* (Ugolini *et al.*, 1997), and when infused into Rhesus macaques (*Macaca mulatta*) they can protect against vaginal transmission of pathogenic SIV / HIV-1 chimeric (SHIV) virus. However, epidemiological studies

have suggested that an overall high anti-gp120 antibody response correlates with rapid disease progression (Rusconi *et al.*, 1998) although the presence of antibodies to the V3 loop of gp120 correlate with protection from progression (Juompan *et al.*, 1998). Slow disease progression is predicted by a strong CTL response and antibodies to the Gag proteins (Klein *et al.*, 1995; Rusconi *et al.*, 1998). Many vaccines currently being developed and tested (for example, Kelleher *et al.*, 1997) are based on the V3 loop of gp120 because this area of gp120 contains the principle neutralisation domains (Juompan *et al.*, 1998) and can be antigenically mimicked by synthetic peptides (Hart *et al.*, 1990). Attempts at mimicking antigenic epitopes in gp120-based peptides have focused on continuous epitopes with relatively little work on discontinuous epitopes.

IgA-mediated mucosal immunity may prove to be more important in protection from sexual transmission than IgG seroconversion (Mazzoli *et al.*, 1997; Zagury *et al.*, 1998; Kaul *et al.*, 1999). It would be expected that a vaccine against sexual transmission of HIV-1 would be required to induce a mucosal response, so the use of mucosal vaccine delivery and mucosal adjuvants such as cholera toxoid need to be investigated (Velin *et al.*, 1998).

Using peptides as vaccines has the advantage that the induced immune response can be closely tailored to defined epitopes. Recombinant peptides have been used successfully as vaccines against hepatitis B (Parfitt, 1999) and several trials of recombinant AIDS vaccines are currently taking place (Kelleher *et al.*, 1997; Boily *et al.*, 1999).

Early work on synthetic gp120 peptides

The interaction of CD4 and gp120 is complex and leads to conformational changes in gp120, gp41 and possibly CD4 (Verrier *et al.*, 1997). Point mutation and epitope mapping studies (Olshevsky *et al.*, 1990; Thali *et al.*, 1991) identified five conserved discontinuous residues of gp120, which form part of the CD4 binding site. More recent evidence (Wyatt and Sodroski, 1998b) from crystallographic studies confirms the importance of these residues in CD4 interactions and shows that they surround a deep cavity, which interacts with F43 on CD4. D368 and E370 in the C3 domain of gp120 and D457 in the C4 domain (some authorities place D457 just inside the V5

domain) are critically important for CD4 binding. Because these residues are near conserved cysteine residues (C378 and C445) it was suspected, long before crystallographic confirmation, that although discontinuous, these residues would be spatially proximal. Figure 6.1 shows the structure of gp120 in this region and how the important residues become close.

The work on discontinuous gp120 peptide sequences reviewed below was carried out in the University of Edinburgh's Departments of Pathology and Chemistry from 1996 to 1998 (Cotton et al., 1996; Heslop, 1997; Howie et al., 1998). See figure 6.1 and table 6.1 for details of peptides discussed and the abbreviation list at the start of this thesis for the standard, single letter amino acid abbreviations used here.

The synthesis of GC-1 (figure 6.1) represented the first deliberate attempt to mimic, within a linear peptide, the structure of a discontinuous region of a protein by anchoring the structure around a disulphide bond. Circular dichroism measurements indicate that the configuration of GC-1 and the related GC-2 peptide is dependent on oxidation of the cysteines and probably involves a hydrophobic association between residues 376, 377, 446 and 447 (Cotton *et al.*, 1996). The observation that antibodies raised in mice against GC-1 fail to recognise GC-2, but do recognise reduced GC-1 and linear peptides representing single continuous 'arms' of GC-1 (Cotton *et al.*, 1996) supports the theory that the hydrophobic region close to the C-C bond is not exposed on the peptide surface. Anti-GC-1 is able to bind GC-1 better than mixtures of peptides representing the 'arms' either side of the C-C bond. This suggests that some of the epitopes recognised by anti-GC-1 contained components from both 'arms' of GC-1, that is, they are discontinuous. Anti-GC-1 is able to bind to shared epitopes on gp120 indicating at least partial mimicking of gp120 conformation (Cotton *et al.*, 1996). GC-1 is able to bind to CD4 on transfected HeLa cells much better than either of the peptide 'arms' or GC-2 (Cotton *et al.*, 1996), indicating that as well as retaining some of gp120's discontinuous antigenic epitopes discontinuous CD4 binding motifs are also retained. GC-1 is able to block CD4 / gp120 interactions, giving the first indication of its possible therapeutic role.

GC-1 analogues

In order to confirm their importance in GC-1's antigenicity and CD4 binding properties, the E370 and D457 residues of GC-1 were replaced by alanines in analogues of GC-1. Analogue 3.5 contains a D→A457 substitution and analogue 3.6 contains a D→A457 and an E→A370 substitution. These substitutions resulted in peptides that could not efficiently bind CD4 or be recognised by anti-GC-1 (Howie *et al.*, 1998). A further peptide, 3.7 (originally called IH-1), was prepared (figure 6.1, Howie *et al.*, 1998). 3.7 incorporates an additional 12-residue branch, which was also synthesised separately as peptide 4.3 (figure 6.1). This extra branch is an additional region of gp120's C4 domain which contains residue W427, important for CD4 binding and Q422 and K421, involved in CCR-5 binding and M-tropism (Howie *et al.*, 1998). Although peptide 4.3 does not have any sequence homology with α chemokines, 4.3's Hopp and Wood hydropathy values and residue molecular weights are similar to those for RANTES, MIP-1α and MIP-1β, suggesting that 4.3 could be CCR-5 binding (Howie *et al.*, 1999). Antibody cross-reactivity studies show that 3.7 shares epitopes with GC-1 but also contains additional immune reactivity. 3.7 binds to the $CD4^+CCR5^+$ T-cell line H9, better than GC-1 (Howie *et al.*, 1998). This data suggests that 3.7 is a better R5-tropic gp120 mimic than GC-1. 3.7 binds to the monocytic cell line MM6 and colocalises with CD4 (Howie *et al.*, 1999). 3.7 is also able to bind to CCR-5 on MM6 cells, as shown by its inhibition of fluoresceinated MIP-1α binding (Howie *et al.*, 1999). 3.7 is likely to bind to the same CDR2 site on CD4 domain 1 as gp120, because it is able to inhibit the binding to H9 cells of gp120-competing anti-CD4 monoclonal antibody (clone QS4120), but not non-gp120-completing anti-CD4 (clone L120) (Howie *et al.*, 1999). QS4120's epitope has been mapped to CDR2 of CD4, whereas L120 binds to domain 4 of CD4 (Wyatt *et al.*, 1998a; Chen, 1998a).

Biological activities of synthetic peptides

Gp120 is able to cause activation-induced cell death (AICD) in $CD4^+$ T-cells (Meyaard *et al.*, 1992; Groux *et al.*, 1992; Gougeon *et al.*, 1993a; Gougeon and Montagnier, 1993b; Howie *et al.*, 1994). AICD is mediated by the transduction of

signals through CD4 (LaurentCrawford *et al.*, 1993; Wang *et al.*, 1994; Maldarelli *et al.*, 1995; Corbeil and Richman, 1995). The signal from gp120 is usually only strong enough to induce apoptosis when the gp120 is cross-linked by antibodies (Howie *et al.*, 1998). GC-1 was able to mimic gp120 by inducing H9 cell apoptosis when cross-linked by anti-GC-1. GC-1 alone was able to inhibit gp120 / anti-gp120-induced apoptosis by competing with gp120 for CD4 binding (Howie *et al.*, 1998). Work presented in this thesis tested some of these synthetic peptides in an HIV-1 infection assay for the first time.

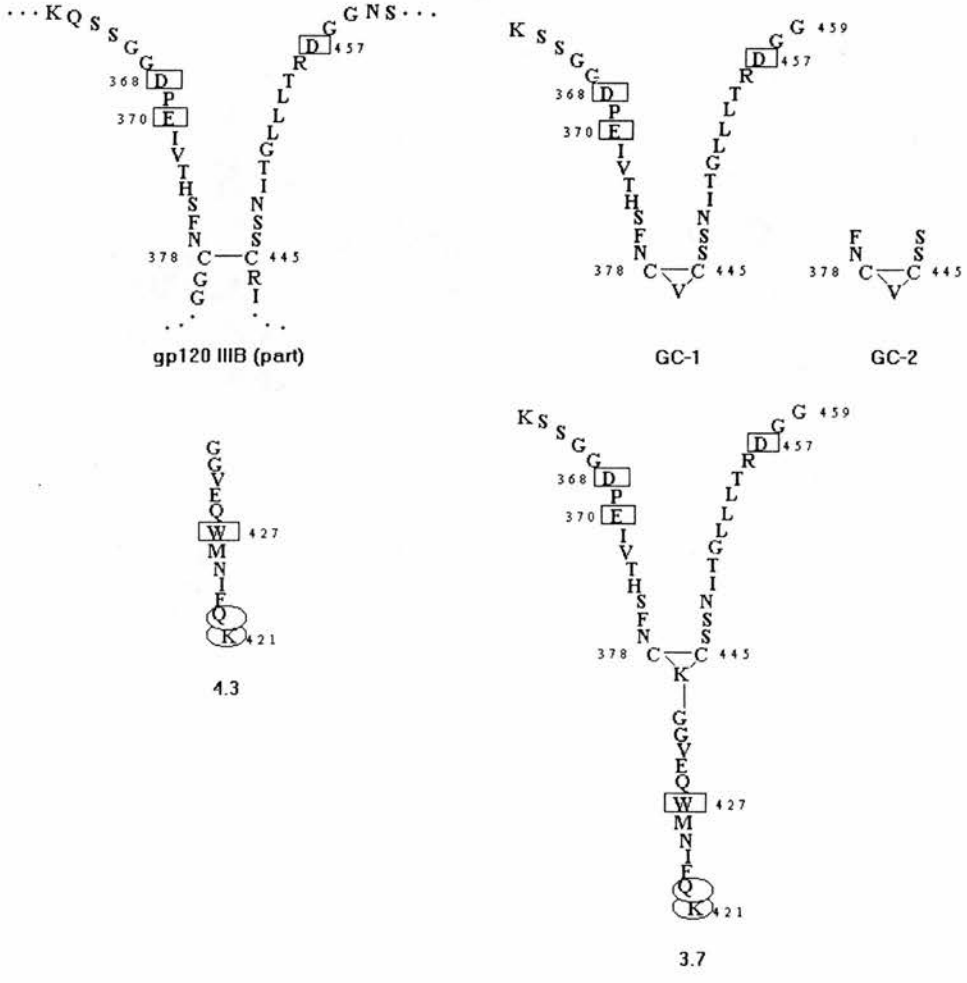


Figure 6.1. Schematic representation of an area of gp120 and synthetic peptides based on its structure. Boxed residues are critical for CD4 binding. Circled residues are involved in M-tropism and CCR-5 binding. The

first diagram shows an area of $gp120_{IIIB}$. The sequence and to some extent the secondary structure of this part of $gp120_{IIIB}$ has been reconstructed in the peptide GC-1, which also has an extra lysine residue (K363) to give the option of coupling to a carrier protein. GC-2 represents a small part of GC-1. Peptide 4.3 is based on another part of gp120, this time incorporating residues required for CD4 and CCR-5 binding. GC-1 and 4.3 are combined in 3.7, this requires the central valine to be substituted by a lysine. Residue numbering is based on that for the $HIV\text{-}1_{IIIB}$ sequence (SwissProt accessions P03376 and P04624, Crowl *et al.*, 1985; Muesing *et al.*, 1985).

Figure adapted from Howie *et al.*, 1999, and Cotton *et al.*, 1996. © The FASEB Journal, 1999 and © Elsevier Science Ltd, 1996.

Protein / peptide	Number of residues	Molecular weight / D
gp120	~510	120,000
GC-1	32	3,200
GC-2	7	700
3.5	32	3,200
3.6	32	3,200
3.7	44	4,500
4.3	12	1,400
PSS023	34	3,500

Table 6.1. Properties of gp120 and gp120 derived peptides. PSS023 is a random linear 34-mer control peptide.

HIV-1 infectivity assays

Several different *in vitro* assay systems have been used to test potential anti-HIV-1 drugs (Mohan and Baba, 1995). These include:

- protection of target cells from cytotoxic effect of HIV-1 (Mitsuya *et al.*, 1985)
- inhibition of HIV-1 induced syncytia formation / syncytial plaque forming assay (Colligan *et al.*, 1993)
- inhibition of p24 (Gag) protein production (Mohan and Baba, 1995)

- inhibition of reverse transcriptase production (Mohan and Baba, 1995)
- inhibition of production of viral DNA of RNA (Mitsuya *et al.*, 1987).

Several factors influence the choice of assay, not least the ease of carrying it out under category-three pathogen-containment conditions. The choice of cell type to use in the assay is important; the work described below used primary macrophages as the HIV-1 target cell. Although earlier work on GC-1 and 3.7 used cell lines, it was felt that a primary cell culture might model *in vivo* APC infection more closely. With an interest in APCs in general, either macrophages or dendritic cells could have been used. Macrophages were chosen as they could be grown from blood monocytes more easily and cheaply and without the need for the exogenous cytokine GM-CSF which is known to effect HIV-1 replication rates (Crowe and Lopez, 1997; Kedzierska *et al.*, 1998) and could complicate the assay. It was decided to use HIV-1_{BAL} as the viral strain to test the peptides against. BAL is an M-tropic, CCR-5 utilising HIV-1 strain (Gartenhaus *et al.*, 1991) and because some of the peptides to test had CCR-5 binding activity, this was thought to be most appropriate. Primary isolates of HIV-1 could have been used in the assay, but these would not have been available in large amounts and would have introduced an additional source of variability. The use of HIV-1_{BAL} avoids the problems associated with T-cell line adapted HIV-1 strains which have been shown to give misleading results in some studies due to their unusually high CD4 binding affinity (Daar *et al.*, 1990).

It has been reported (Mohan and Baba, 1995) that the viral inoculum size (expressed as MOI or $TCID_{50}$) used in an assay can influence the results. The need to calculate MOI precisely was removed in the studies presented here by using HIV-1_{BAL} from the same single supernatant batch in each experiment and by adding a large amount of HIV-1 to the cells in each case so that a ratio of virus particles to target cells of at least 100:1 and a correspondingly high MOI could be assumed. Using an excess of virus brings the danger that subtle anti-viral effects could be missed. This problem was not fully addressed in our studies. However, allowing the peptides to be pre-incubated with the target cells in the absence of HIV-1 gave them a 'head start' on the virus and enabled them to demonstrate anti-viral properties which might otherwise have been swamped by HIV-1 excess.

High tissue culture protein concentrations (from high concentrations of human or bovine serum) have been shown to affect the anti-viral activities of some experimental compounds (Kageyama *et al.*, 1994). Therefore, the assay system in the studies described here used medium containing only 5% human serum as required for macrophage survival. The assay system used also allowed the peptides tested to pre-incubate with the macrophages for a period in the absence of serum.

The HIV-1 infection assay chosen for these studies identifies infection of target cells by using semi-quantitative RT-PCR to specifically detect HIV-1 mRNA. The RT-PCR reaction used was designed not to detect either HIV-1 genomic RNA or DNA provirus, but only appropriately spliced mRNA. Such a RT-PCR based assay would be ideally suited to the real-time PCR techniques used elsewhere in this thesis. However, PE Biosystems (manufacturer of the TaqMan™ real-time PCR system and holders of exclusive patent rights to this technology, Gelfand *et al.*, 1993) prohibit the use of their system for the detection of HIV-1, even for pure research purposes.

An RT-PCR based infection assay has the advantage over some other techniques in that a minimum of time needs to be spent in the category-three containment facilities because the extracted DNase treated RNA is considered safe to handle in an ordinary laboratory if good laboratory practice (GLP) is observed. RT-PCR is more rapid than techniques which involve counting cells by eye, and unlike plaque-forming assays it can be used with NSI HIV-1 strains. When interpreting results from an assay which measures mRNA it needs to be born in mind that in addition to measuring infection efficiency, transcription efficiency may also influence results.

Aims of chapter

- To validate, by variable cycle RT-PCR the infection assay system chosen for this study and to determine the optimum number of cycles to use.

- To investigate if the gp120 based peptides described above are able to block HIV-1 infection of macrophages.

- To use infection assay data to comment on possible improvements to the design of these peptides.

Methods

See chapter 2 for details of procedures.

Synthesis of peptides

Peptides GC-1, 3.7, PSS023 and 4.3 were synthesised by Albachem Ltd (Edinburgh) and the University of Edinburgh Department of Chemistry using the F-moc system as described above, in Cotton *et al.*, 1996, and Howie *et al.*, 1998. The reactions were carried out on an Applied Biosynthesis 430A automated peptide synthesiser (Parke-Davis, Warner-Lambert, Eastleigh, Hampshire, UK) fitted with an Applied Biosystems 757 UV monitoring system (PE Biosystems) allowing real-time monitoring of coupling efficiencies. Briefly, GC-1, PSS023 and 4.3 were synthesised entirely automatedly from the carboxyl to amino terminals. The cysteine residues in GC-1 were oxidised in air to form the disulphide bridge between residues 378 and 443. The synthesis of 3.7 was more complex and involved automated G459 to C445 synthesis, lysine that had been protected at its N^{α} amino function by 4,4-dimethyl-2,6-dioxocyvlohex-1-ylidine (Dde) was then manually coupled and automated synthesis restarted from the N^{ε} amino function to K421. After removal of Dda in hydrazine, synthesis was continued from the N^{α} amino function to K363. The cysteines were then oxidised in air.

Infectivity assay

The agent under test was added at 30μM (except gp120, which was used at 0.3μM) to the infection assay system described in chapter 2. Peptide PSS023 was used as a control and has no sequence homology to gp120. It is a random 34-mer peptide (Howie *et al.*, 1999).

The semi-quantitative RT-PCR reaction used to detect HIV-1 mRNA must be optimised and validated before it can be used to generate meaningful results. The appropriate number of PCR cycles for the reaction needs to be chosen so that an HIV-1 and *β-actin* housekeeping control signal can both be detected, but neither signal becomes saturated. This allows both increases and decreases of starting mRNA to be detected as increases or decreases in band intensity. Briefly, the RT-PCR reaction described in chapter 2 was run with a known positive for HIV-1. 0.1μg of positive control RNA was used for each reaction tube because this was the amount of RNA reverse-transcribed for the test samples. The PCR reaction was set to run for 50 cycles and individual tubes were removed from the thermal cycler after undergoing 25 to 50 cycles each (see figure 6.2 for exact number of cycles used). The samples were then electrophoresed on an agarose gel, stained with ethidium bromide and UV illuminated. The band intensity was assessed visually and using the Enhanced Analysis System (EASY, version 4.19, Scotlab).

Results

RT-PCR validation

Figure 6.2 shows the data obtained from the variable cycle RT-PCR experiment. 32 was chosen as the number of cycles for all future semi-quantitative RT-PCR under similar conditions.

Infectivity assay

Recombinant R5-tropic gp120 at 0.3μM completely blocked detectable *in vitro* infection of macrophages after challenge with HIV-1_{BAL} for both 72 and 94 hours (figure 6.3). The irrelevant peptide PSS023 at 30μM had no effect on the infectability of macrophages (data not shown). At this concentration 3.7 and its analogues blocked infection to varying extents. Figure 6.3c shows the infection levels detected in macrophages that were pre-treated with various peptides before HIV-1_{BAL} challenge, expressed relative to cells treated with PSS023. 3.7 and 4.3 were approximately equally effective at blocking HIV-1 infection, showing a 71%

and 83% respective reduction in infection level of macrophages after 72 hours of HIV-1_{BAL} challenge. The protective effect of 4.3 was, however, substantially lost by 94 hours with only an approximately 29% reduction in infection observed. The protection offered by 3.7 was more long lasting, by 94 hours the reduction in macrophage infection caused by this peptide only fell from 71% to 62%.

GC-1 treatment of cells caused an enhancement of infection. At 72 hours this enhancement was slight with an infection level of 121% of the control value. By 94 hours the macrophage infection level was enhanced to 243% of the control value.

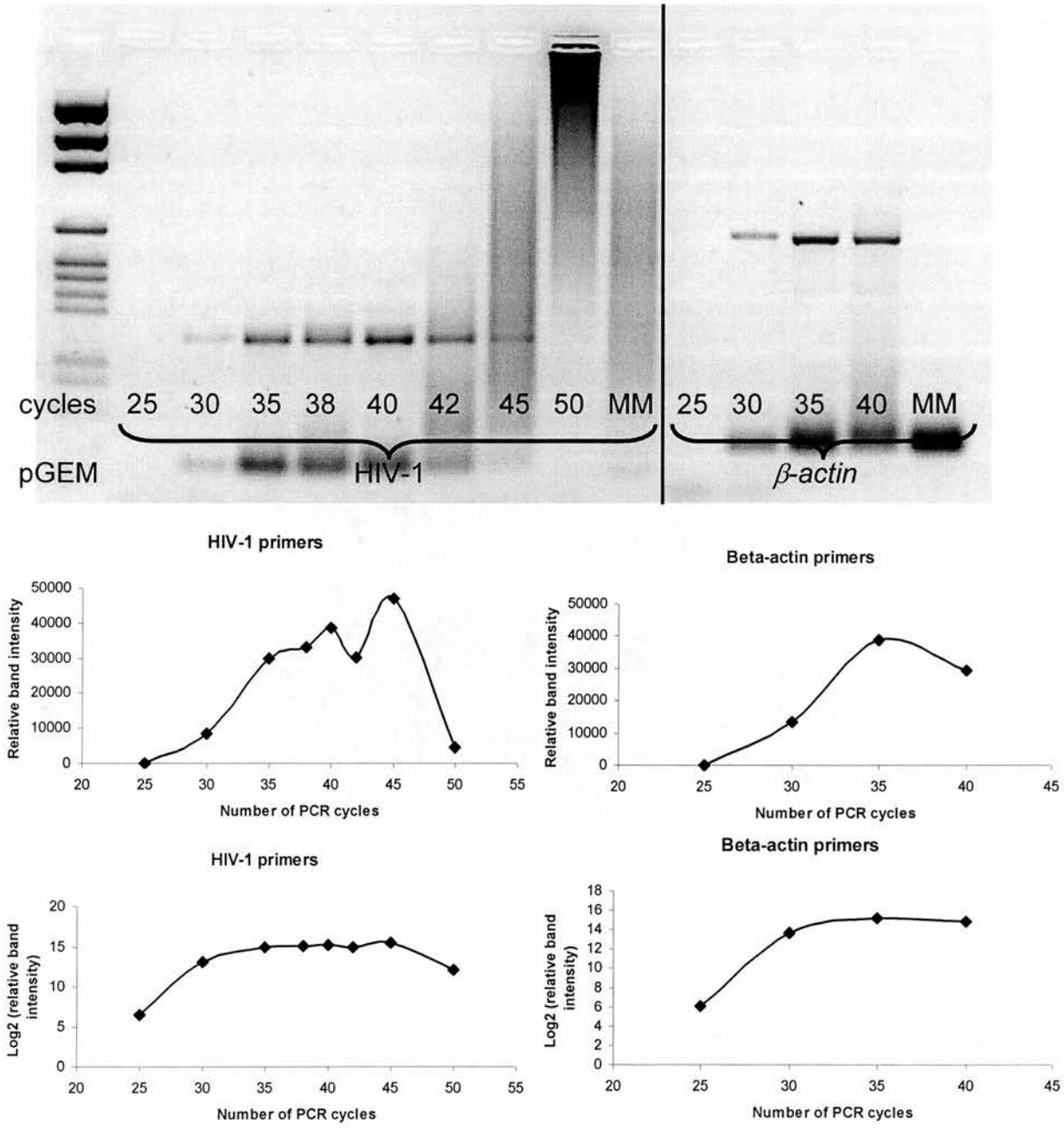


Figure 6.2. Variable cycle RT-PCR. The electropherogram shows PCR product bands (*β-actin* at 661bp and *HIV-1* at 215 bp) obtained by amplification of an equal amount of starting material for the number of cycles and primer sets indicated. It can be seen that with increasing cycle number the band intensity at first increases and then declines as non-specific reactions, nucleic acid degradation and enzyme denaturation increase. Accompanying graphs show results of image analysis on the electropherogram, band intensity and its base-two logarithm is plotted against number of cycles. 32 cycles is found on a region of the graphs where the band intensity signal is still increasing.

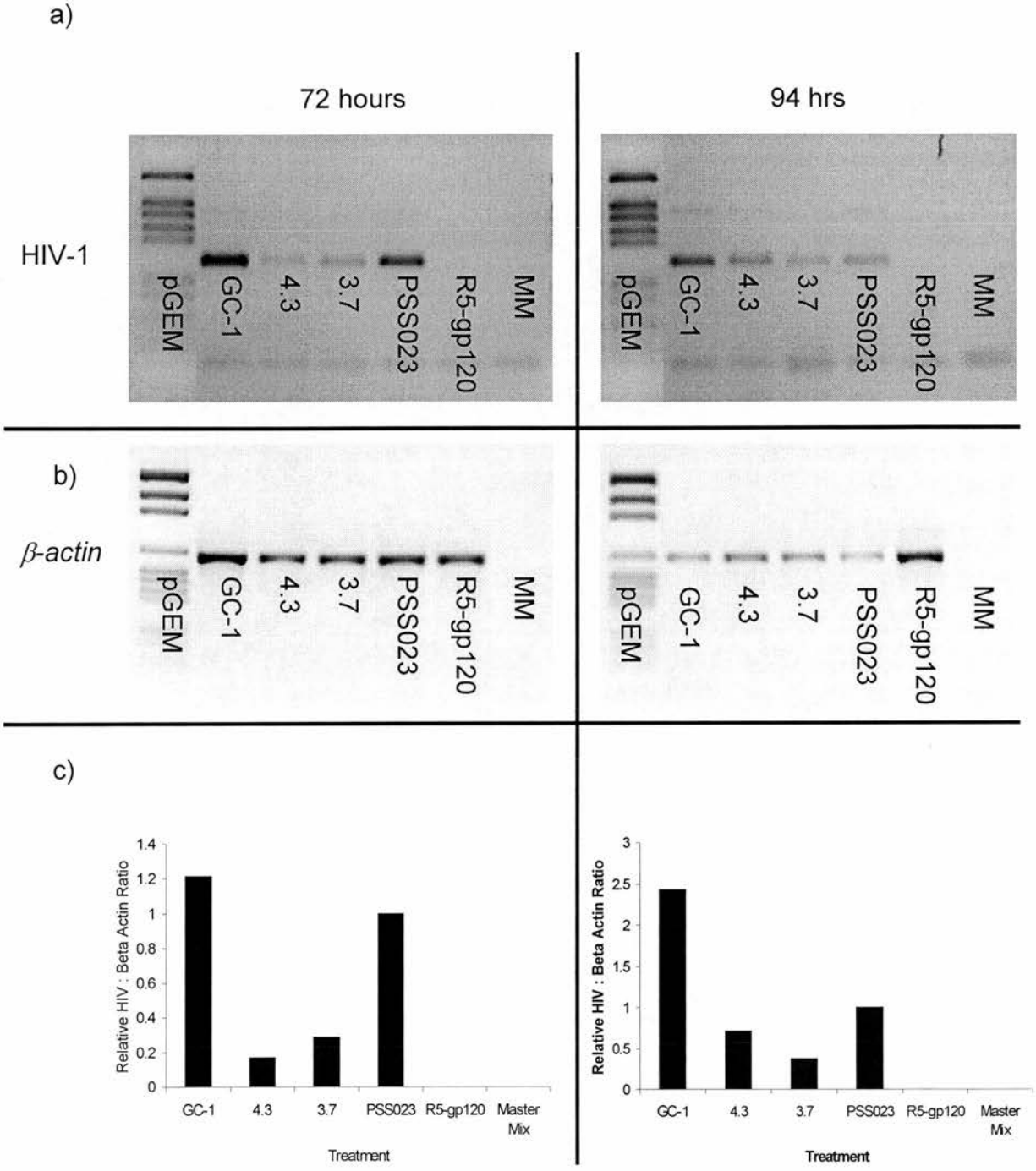


Figure 6.3. Results of infectivity assay. RT-PCR was carried out on RNA from cells treated with the proteins or peptides indicated and then challenged with HIV-1_{BAL} for 72 hours (left panels) or 94 hours (right panels) in order to semi-quantify mRNA for the *β-actin* housekeeping gene (part b) and HIV-1 (part a). The electropherograms show raw data from a typical experiment. The graphs (part c) show the mean band intensity ratios from two similar experiments. MM = master mix (negative control).

Discussion

Because 3.7 can bind to both CD4 and CCR-5 we tested the ability of 3.7 and two of its analogues to inhibit infection by an M-tropic HIV-1 strain. Using primary, peripheral blood-derived macrophages we found that 3.7 could indeed inhibit infection with HIV-1_{BAL} whereas the irrelevant peptide PSS023 had no effect. This is promising if 3.7 is to be considered as a therapeutic candidate. However experiments with GC-1 and 4.3 highlight some important issues and potential pitfalls of therapeutic peptide design which must be addressed and could be important in guiding the design of future generations of synthetic peptides.

Stability of peptides

The protective effect of full length R5-tropic gp120 lasted for at least 94 hours; the protection from infection offered by 4.3 and 3.7 declined from 72 to 94 hours (by 65% and 13% respectively). Gp120 was assayed at a different molarity to the peptides and this could have influenced the longevity of the infection inhibition effect. Additionally gp120 may have been protective against infection by inducing a decline in macrophage surface CD4 and CCR-5 as described in chapter 4. An alternative and plausible explanation is offered by differential peptide and protein stability in tissue culture condition, and may be related to the *in vivo* stability of these molecules. In tissue culture conditions (and *in vivo*) proteins will be exposed to the degradative effects of extracellular proteases and the endocytic properties of macrophages. The peptides and proteins in this study may have been protected from breakdown by their secondary structure or by complexing with CD4 or CCR-5. In this study gp120 was the most stable molecule, followed by 3.7 and then 4.3. The greater stability of 3.7 over 4.3 may have been due to its higher affinity for cellular receptors (Howie *et al.*, 1998), or its increased gp120-mimicing secondary structure. It would be possible to assay peptides in cell culture supernatant and in the circulation of human volunteers in order to measure their half-life. Using novel, branched peptide synthesis techniques to allow the retention of more protein secondary structure may not only be beneficial from a therapeutic viewpoint by

preserving the biological activity of the parent protein, it may also increase the molecular stability of the peptide.

Blocking efficiency

Although 4.3 appears to be less stable in culture than 3.7 (see above and figure 6.3) 72 hours after HIV-1_{BAL} challenge, 4.3 was able to block infection just as well, if not better than 3.7. This is despite the fact that 4.3 contains none of the three CD4-binding residues of GC-1 (figure 6.1). The reason for 4.3's greater activity could be that the K363-C378 and C445-G459 arms of 3.7 spatially hindered the important infection-blocking K421-K433 residues, which were freer to interact with cellular targets in 4.3. An alternative explanation, partially supported by observations of the effect of GC-1, is that the K363-C378 and C445-G459 arms of 3.7 may enhance infection (see below). It is theoretically possible that instead of blocking infection, the peptides were simply killing the cells thereby removing potential infection targets. This seems highly unlikely as no noticeable difference was observed between treated and untreated cells when closely examined by phase-contrast microscopy. A more vigorous approach to assessing cell death was taken in chapter 5 where it was shown, by annexin V staining, that in the absence of infection R5-gp120 did not induce apoptosis in macrophage cultures.

Infection enhancement

GC-1 enhanced the infection of macrophages by HIV-1_{BAL}. There is a precedent for anti-CD4 (Stamatatos *et al.*, 1997), sCD4 (Demaria and Bushkin, 1996) and CD4 binding synthetic peptides based on the V3 loop of gp20 (Dettin *et al.*, 1998) enhancing infection. The mechanism of infection enhancement by GC-1 is likely to be the induction of conformational changes in CD4 or gp120 which result in gp120 being able to bind to CD4 and CCR-5 with greater efficiency (Jones *et al.*, 1998; Choe *et al.*, 1998; Dettin *et al.*, 1998). An alternative explanation is that GC-1 did not enhance HIV-1 entry, but rather the action of this molecule on already infected cells increased the transcription rate of HIV-1 mRNA, giving an increased signal in the assay used (Tremblay *et al.*, 1994; Morio *et al.*, 1997; Briant *et al.*, 1998). The

observation that GC-1 can enhance HIV-1 infection highlights a serious risk in the design and use of viral entry inhibitors. Previous studies have shown that GC-1 is able to bind to CD4 but not with the same affinity as 3.7 (Howie *et al.*, 1998; Howie *et al.*, 1999). One might imagine that weakly binding peptides (e.g., GC-1, 3.5, 3.6) might be more likely to cause infection enhancement than more strongly interacting peptides (e.g., 3.7). Weakly binding ligands would be able to 'coax' the target proteins into conformational changes but then fall off their targets rather than bind so tightly so as to block gp120 / host protein interactions. The data in figure 6.3 could also be interpreted to suggest that infection enhancement is a risk when a peptide contains CD4-binding residues alone (e.g., GC-1) but not when both CD4 and CCR-5 binding residues are present (e.g., 4.3, 3.7, and gp120). It is important to remember that infection enhancement *in vitro* may not accurately model the potential *in vivo* situation; viral tropism, viral and peptide concentration and cellular activation states may be different.

3.7 as an vaccine

Experiments described in this chapter's introduction have demonstrated that 3.7 and GC-1 contain discontinuous and continuous BALB/C murine B-cell epitopes (Chen, 1998a; Howie *et al.*, 1999). The fact that antibody to these peptides was all of the IgG class (Weissman *et al.*, 1995) means that there must have been isotype switching during the anti-peptide immune response. This implicates T-cell involvement and is evidence that the peptides contain murine T-cell epitopes. Whether or not 3.7 contains human T- and B-cell epitopes is not demonstrated by its immunogenicity in mice because of species differences between the two immune systems. However, the mouse studies at least show that the peptides are capable of being processed into an immune stimulatory form. Predicting immunogenicity in humans is complicated by different class I and II MHC restriction of T-cell epitopes in individuals of an outbred population. However, comparisons of the 3.7 sequence with published epitopes from the HIV molecular immunology database (figure 6.2 and Katz *et al.*, 1995) shows that 3.7 contains several previously identified T- and B-Cell epitopes. On consideration of the above data, it seems likely that 3.7 would be immunogenic if humans were vaccinated with it. If 3.7 were administered as a vaccine, its

immunogenicity would be advantageous, especially if 3.7 contains neutralising epitopes (the B-cell epitope listed in table 6.2 is neutralising, Lasky *et al.*, 1987). However, if 3.7 were to be used as a post-exposure prophylactic to block infection of cells after an individual had exposed themselves to risk, the induction of B-cell immunity would be disadvantageous. An antibody produced against the prophylactic peptide could bind to the peptide and neutralise its action; this would reduce the effectiveness of the peptide if used a second time.

3.7 region and sequence			Reference
364 to 378	421 to 433	445 to 459	
KSSGGDPEIVTHSFNC	KQFINMWQEVGG	CSSNITGLLLTRDGG	
Continuous antibody epitopes			
	QFINMWQEVK		Lasky *et al.*, 1987
T-helper epitopes			
QSSGGDPEIV			Schrier *et al.*, 1989
SSGGKPEIVTHSFNC			Wahren *et al.*, 1989
		SSNITGLLLTRDGGTC	Manca *et al.*, 1995
CTL epitopes			
PEIVTHS			Dadaglio *et al.*, 1991

Table 6.2. Alignment of 3.7 with human T- and B-cell epitopes. Details of epitopes were obtained from the HIV molecular immunology database (Korber *et al.*, 1998) and references cited in the table. Discontinuous B-cell epitopes are not shown. Imperfect residue matches are underlined.

Future directions

That the synthetic peptide 3.7 derived from three discontinuous sequence stretches of conserved regions can adopt a structure which allows it to interact with cell surface ligands of native gp120 and partially inhibit infection of primary macrophages has implications for the development of both therapeutic interventions and a synthetic vaccine. The approach of using synthetic chemistry to bring together in a peptide regions of a protein around a C-C bond in order to preserves discontinuous antigenic epitopes or binding motifs also has more general implications for the synthesis of

novel peptides representing complex, sequence discontinuous, ligand binding sites of important biological proteins.

The HIV-1 infection enhancing effect of GC-1 serves as an important reminder of this risk when considering the design of therapeutic peptides. It would be useful to investigate this phenomenon more closely in an attempt to define which gp120 regions are involved in enhancement and which in inhibition of infection. This information would be useful in guiding the design of future peptide-based HIV-1 entry inhibitors.

The observation that 4.3 is able to show similar anti-infection properties to 3.7 raises the possibility that the complex and expensive synthesis of branched peptides may not be necessary. 4.3 is a short linear peptide and as such can be made cheaply by synthetic or genetic engineering techniques. Since 4.3 was designed, the residues of gp120 involved in CCR-5 binding have been better defined (see figure 6.4 and Rizzuto *et al.*, 1998). Additional residues in the C4 region of gp120 on which 4.3 was based have been identified as important for CCR-5 binding. These residues include R419, Q422, I423, I424, M426 and E429 (Rizzuto *et al.*, 1998). It would be possible to produce a series of peptides based on the success of 4.3 but incorporating these recently identified residues. A combinatorial chemistry approach could be used to generate a large series of 4.3-based molecules for testing in an infectivity assay. If the therapeutic target of this work is to produce a peptide for use as a post-exposure prophylactic, or a vaginal microbicide (The International Working Group on Vaginal Microbicides, 1996) it is important to use a peptide with low immunogenity. Only one immunogenic epitope has been described in 4.3 (see Lasky *et al.*, 1987, and figure 6.3). It is possible that if combinatorial chemistry were used to produce a series of 4.3 analogues, peptides would be found that efficiently blocked infection but did not result in the production of anti-peptide antibody which would neutralise the infection inhibiting properties of the peptide.

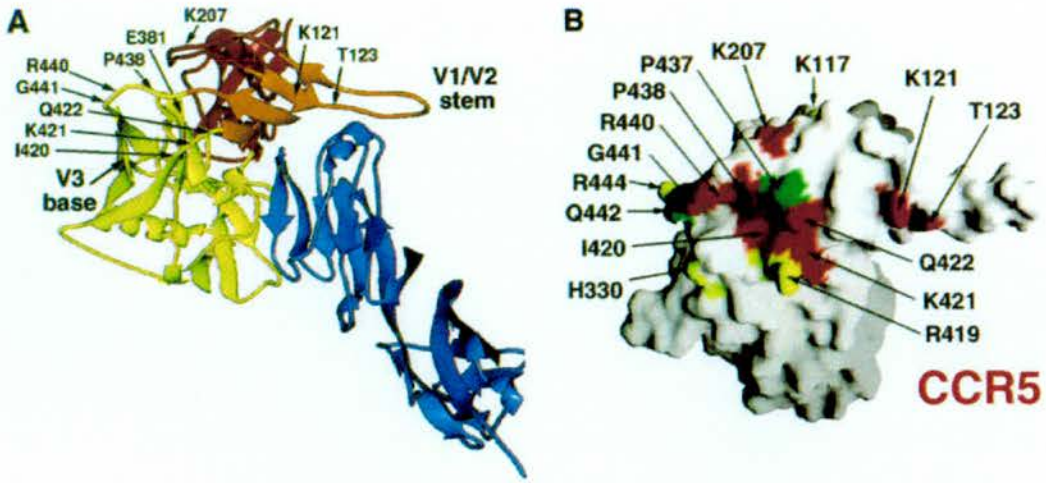


Figure 6.4. Structure of HIV-1 gp120 showing residues implicated in CCR-5 binding. A) Ribbon diagram of gp120 and gp41 (blue). B) Molecular surface diagram of gp120 from the same perspective as A). Coloured residues are associated with CCR-5 binding, yellow indicates residues which when changed result in a ≥ 75% decrease in CCR-5 binding, changes to red residues cause a ≥ 90% decrease in binding and changes to green residues cause a ≥ 50% increase in CCR-5 binding.

Figure from Rizzuto *et al.*, 1998. © The American Association for the Advancement of Science, 1998.

CHAPTER 7

SUMMARY, GENERAL DISCUSSION AND CONCLUSIONS

"And that was the day that we knew, oh! In the world there is a new disease called AIDS. I thought surely this will be the greatest war we have ever fought. Surely many will die. And surely we will be frustrated, unable to help. But I also thought the Americans will find a treatment soon. This will not be forever."

Dr Jayo Kidenya, Bukoba, Tanzania, 1985 (Garrett, 1994).

Summary

This final thesis chapter will re-examine the problems posed by HIV-1, summarise results presented in each of the earlier chapters, and attempt to bring them together and place them in the context of past work and future possibilities.

The major results arising from this work are summarised below:

- Many previous studies have reported the incidence of the *ccr5Δ32* allele. Few have statistically analysed the distribution of this allele. Chapter 3 reports a significant correlation between *ccr5Δ32* allele frequency and northerly latitude, and predicts an allele frequency of 12.61% (8.42% to 16.80% at the 95% confidence level) for a population at the latitude of south east Scotland.

- In a group of 94 anonymous blood donors in the south east of Scotland, the frequency of the *ccr5Δ32* allele was 14.89%. I believe that this is the first report of *ccr5Δ32* incidence in a healthy Scottish population. The allele's incidence fits with that predicted for the population's latitude (chapter 3).

- The M-tropic HIV-1 strain BAL uses CCR-5 as an entry co-receptor. HIV-1_{BAL} was unable to infect homozygous *ccr5Δ32* monocyte-derived macrophages,

which fail to express surface CCR-5. This finding demonstrates that HIV-1_{BAL} macrophage infection requires the use of CCR-5 (chapter 3).

- Gp120 induces loss of macrophage surface CD4 (chapter 4).

- Substantial gp120-induced loss of macrophage surface CD4 is CCR-5 dependent, as it only occurs with M-tropic gp120, not T-tropic gp120, and it does not occur on macrophages homozygous for the *ccr5Δ32* mutation, which fail to express surface CCR-5 (chapter 4).

- Macrophage surface CD4 loss is due to CD4 internalisation by a process resembling receptor-mediated phagocytosis (chapter 4).

- Exposure to M-tropic, but not T-tropic, gp120 reduces an APC's capacity to support T-cell proliferation (chapter 5). This may be because CD4 internalisation (chapter 4) alters antigen processing and presentation efficiencies. Several alternative explanations for reduced T-cell proliferation, involving the Notch pathway and cytokines, were also investigated (see below).

- In addition to M-tropic gp120, seminal plasma extract (SPE) and prostaglandin (PG) treatment of APCs inhibits their ability to support T-cell line proliferation (chapter 5).

- Anergised T-cell lines express elevated levels of Notch ligand mRNA, suggesting the involvement of the Notch pathway in anergy induction (chapter 5), and the possibility that the Notch pathway is involved in the gp120-induced T-cell proliferation defect.

- M-tropic and T-tropic gp120, SPE and PG induce Notch ligand mRNA and down-stream Notch signalling events in APCs. Gp120 induces PG release; this, together with the abolition of gp120-mediated effects by indomethacin, suggests

that the gp120 effects on Notch ligand mRNA expression are mediated (at least in part) by PG (chapter 5).

- mRNA for the Notch pathway signal transduction molecules HES-1 and deltex is found in LC-like DCs but not macrophages. mRNA levels change in LCs following putative Notch signalling. *Deltex,* but not *HES-1*, is expressed in T-cells, but is only modulated in response to gp120-induced events in LCs (chapter 5).

- In addition to the Notch pathway, there is evidence that cytokines and other soluble mediators may be involved in gp120-induced T-cell proliferation defects. TGF-β is released by macrophages and monocyte-derived LCs in response to M-tropic and T-tropic gp120, PG and SPE (chapter 5). An increase in *IL-10* mRNA is also induced by M- and T-tropic gp120, but only after 72 hours – not soon enough to account for gp120 inhibition of T-cell proliferation (chapter 5).

- Synthetic branched peptides based on discontinuous sequences of M-tropic gp120, and retaining some of the antigenic and binding properties of gp120 block M-tropic HIV-1_{BAL} infection of macrophages, and are therefore potential drug candidates (chapter 6).

- However, using gp120-based peptides as an HIV-1 vaccine or other therapeutic agent brings potential dangers. One gp120-based peptide tested caused infection enhancement (chapter 6), and evidence from chapter 5 demonstrates that another gp120-based peptide retains the Notch ligand inducing property of gp120, and therefore, the potential to induce inappropriate tolerance.

Discussion

HIV-1

HIV-1 is a retrovirus which causes AIDS (BarréSinoussi *et al.*, 1983), and infects 40 million people worldwide (WHO 1999 estimate). Following HIV-1 infection, host

immunity declines over several years until the host is killed by a succession of opportunistic infections and/or malignancies (Castro *et al.*, 1993). HIV-1 infects $CD4^+$ T-cells, macrophages and DCs. The first stage of infection is the binding of cell surface CD4 by HIV-1's envelope glycoprotein, gp160 (consisting of gp120 and gp41, Dalgleish *et al.*, 1984). Following CD4 engagement, a conformational change in gp120 reveals residues that bind to one of the chemokine receptors (D'Souza and Harden, 1996). Further conformational changes take place and the viral envelope fuses with the target cell plasmalemma (Pereira *et al.*, 1997; Ji *et al.*, 1999). The range of cell types that an isolate of HIV-1 is able to target is known as its cellular tropism. Cellular tropism is determined by which chemokine receptors a particular isolate's gp120 is able to bind to, and the availability of these receptors on the surface of various cell types (Hoffman and Doms, 1999). HIV-1 can be broadly classified as M-tropic, T-tropic or dual tropic (Berger, 1997). M-tropic virus can infect macrophages, DCs, LCs and T-cells, and utilises CCR-5 as an entry co-receptor. T-tropic virus uses CXCR-4, and can only infect T-cells. Dual tropic HIV-1 has the ability to infect T-cells, macrophages, LCs and DCs via CCR-5 and CXCR-4 (Doranz *et al.*, 1996). Because M-tropic HIV-1 strains such as $HIV\text{-}1_{BAL}$ are limited to using CCR-5 as a co-receptor, individuals who are homozygous for the *ccr5Δ32* mutation and fail to express cell surface CCR-5 are protected from infection by M-tropic strains of HIV-1 (Paxton *et al.*, 1998, and chapter 3). The *ccr5Δ32* mutation confers no phenotype on healthy individuals (Magierowska *et al.*, 1998). It arose approximately 700 years ago (Stephens *et al.*, 1998; Nasioulas *et al.*, 1998) and is common in white Europeans. New results presented in chapter 3 show that the *ccr5Δ32* mutation becomes more common in Europe with increasing northerly latitude, and report the allele frequency for the south east of Scotland, which is predicted by Scotland's northerly latitude. The reasons for the geographic distribution of *ccr5Δ32* are discussed in chapter 5, but remain obscure.

Antigen presenting cells

APCs have a central co-ordinating role in the immune system. Phagocytic APCs act as effectors of the innate immune system. APCs also have a role in regulating the

activity of lymphocytes of the adaptive immune system. In order to be fully activated, T-cells must be presented with MHC-complexed antigenic peptides, and given appropriate co-stimulatory and cytokine signals (Janeway and Travers, 1996). Antigen specific signals delivered to T-cells in the absence of co-stimulation, or in the presence of 'tolerogenic' signals may lead to T-cell anergy (Frauwirth *et al.*, 2000), and/or a regulatory T-cell phenotype (Chen *et al.*, 1994). Any disease that dysregulates APC function, therefore has the potential to disrupt both innate and acquired immunity.

HIV-1, gp120 and APC dysregulation

HIV-1 disease is characterised by a dramatic fall in $CD4^+$ T helper cells, which parallels disease progression. However, the loss of HIV-1 infected $CD4^+$ T-cells does not completely explain the immunodeficiency seen in HIV-1 disease. Immune defects are seen in HIV-1^+ patients before a significant $CD4^+$ T-cell decline, and uninfected T lymphocytes and APCs can be killed or have their function disrupted (Hewson *et al.*, 1999).

Gp120 is arguably the most damaging HIV-1 protein because it is secreted by infected cells, and found in the plasma of HIV-1^+ patients (Oh *et al.*, 1992), where it has the potential to dysregulate the function of uninfected cells. Soluble gp120 retains its ability to bind to the cellular receptors, CD4, CXCR-4 and CCR-5 (Oh *et al.*, 1992), and the tropism of soluble gp120 influences the range of cell types to which it can bind. HIV-1 infected APCs show a reduced capacity to support T-cell proliferation (Knight *et al.*, 1997a). Chapter 5 shows that treatment of uninfected APCs with M-tropic, but not T-tropic, gp120 reduces their capacity to support the proliferation of a T-cell line. SPE or PGE_2 treatment results in a similar proliferation deficit. Several mechanism (discussed below) could account for this. HIV-i is able to cause changes to APC cytokine production (Ankel *et al.*, 1996; Gessani *et al.*, 1997), and can influence the Th1 – Th2 balance of an immune response (Clerici and Shearer, 1993). HIV-1 proteins can mimic and antagonise cytokines (Idziorek *et al.*, 1998; Howie *et al.*, 1999) and change MHC (Peter, 1998), CD4 (Willey *et al.*, 1992; Rhee and Marsh, 1994), Fas ligand (Badley *et al.*, 1996; Dockrell *et al.*, 1998), and Fc receptor (De *et al.*, 1998) cell surface levels. Data in this thesis (chapter 5) shows

that from 3 days onwards *IL-10* mRNA in macrophages is up-regulated by exposure to gp120. It is possible that gp120-induced IL-10 could lead to immune deviation. However, no gp120-induced IL-10 release was detected by ELISA at earlier time-points. This rules out IL-10 mediated immune deviation as a cause of the observed gp120-induced T-cell proliferation deficiency.

Gp120 and CD4

There are several reports of T-tropic gp120 inducing a decline in surface CD4 from T-cells (Theodore *et al.*, 1994) and macrophages (Karsten *et al.*, 1996). Research presented in chapter 4 compares the effects of T-tropic and M-tropic gp120 on macrophage surface CD4 levels. It was discovered that M-tropic gp120 induced a far greater CD4 loss than T-tropic gp120 (Hewson and Howie, 1998, chapter 4). Chapter 4 presents results from experiments comparing only two gp120s, that from the prototypic T-tropic HIV-1_{IIIB}, and one from a primary M-tropic patient isolate. It would be interesting to compare a wider range of gp120 samples in order to strengthen the hypothesis that differential chemokine receptor usage by gp120 determines the extent of induced CD4 loss. Further proof that CCR-5 binding is required for substantial CD4 loss comes from the observations that CD4 loss was not observed in *ccr5* null macrophages, and *ccr5* mRNA is up-regulated in response to CD4 loss (implying replenishment following a concomitant CCR-5 loss). It seems likely that substantial M-tropic gp120-induced surface CD4 loss is due to the triggering of receptor mediated phagocytosis. Confocal microscopy (chapter 4) showed that M-tropic but not T-tropic gp120 was internalised as large vesicles resembling those formed during receptor-mediated phagocytosis. Further work could confirm this hypothesis by investigating the effect of pharmacological phagocytosis inhibitors on CD4 loss. It would also be interesting to know if the receptor mediated phagocytosis of CD4 / gp120 / CCR-5 complexes is macrophage specific, and what signal transduction events are involved in triggering it. It is not known to what extent CD4 and CCR-5 mediated phagocytosis of gp120 occurs *in vivo*. One indirect method of measuring this would be to obtain lymph node biopsies from AIDS patients and look for increased levels of intracellular CD4 protein or mRNA, which would indicate chronic CD4 loss and renewal.

Surface CD4 and CCR-5 loss has several potential consequences for macrophage function. Chemotactic responses to IL-16 and MIP-1α may be lost. M-tropic gp120 endocytosis leads to increased intracellular pools of CD4, gp120 (and presumably CCR-5, chapter 4). Although these proteins have been detected in HLA DR-containing intracellular compartments, the compartments in which they are located could be defined in more detail in order to deduce the likely fate of these potentially antigenic proteins. Increased efficiency of processing and MHC presentation of these host and viral proteins could have consequences for the production of protective and autoimmune responses. There is also the danger that chronically increased levels of MHC-loading with CD4, gp120 or CCR-5 may prevent other antigenic peptides being presented at sufficiently high densities to trigger T-cell activation. Such a mechanism could explain the reduction in the capacity to support T-cell proliferation seen in APCs incubated with M-tropic gp120. M-tropic gp120 induced surface CD4 loss within 1 hour, fast enough to account for the proliferation defect. The strain specificity of the CD4 loss fits with the observation that gp120-induced T-cell proliferation decreases are also strain specific (chapter 5).

Gp120 and inappropriate tolerance

An alternative explanation for the immune deficit seen in uninfected T-cells of HIV-1^+ patients, and the actions of M-tropic gp120 on the ability of APCs to support T-cell proliferation could be that HIV-1 subverts the physiological mechanisms of tolerance induction so that APCs give tolerogenic, rather than activatory, signals to T-cells concomitant to an antigen-specific signal. It is known that gp120 can trigger APCs to produce the immunosuppressive mediators IL-10 (Borghi *et al.*, 1995) and PGE (Denis, 1994). There is a precedent for human viruses to induce inappropriate immune tolerance and suppression (Karp *et al.*, 1996; Nokta *et al.*, 1996; Alcami and Koszinowski, 2000), and it may be easier for HIV-1 to do this when transmitted in semen because of the immunosuppressive / tolerogenic cytokines and other soluble mediators contained in seminal plasma (Kelly, 1997). New data in chapter 5 show that gp120 is able to induce TGF-β1 and PGE_2 production by LCs and macrophages

in a non-strain specific way, and that PGE_2- and SPE-treated APCs show a reduced capacity to support T-cell proliferation. Indomethacin, a cycloxygenase-2 (COX-2) inhibitor which prevents PG production, can abolish the M-tropic gp120-induced defect in APCs' T-cell proliferation supporting capacity. These observations suggest that the reduced ability of M-tropic gp120-treated APCs to support T-cell proliferation is due to PG (and possibly also TGF-β) release by the APCs. However, PGE_2 is released by APCs in response to gp120 in a non-strain specific fashion, so PGE_2 on its own is unable to account for all features of the APC defect.

HIV prostaglandin and Notch

The Notch pathway has recently been implicated in tolerance induction (Hoyne *et al.*, 2000), and chapter 5 contains data showing that the induction of anergy in T-cell lines causes Notch ligand mRNA to be induced. Chapter 5 presents the first data to show the interaction between Notch signalling and immune modulation by semen or a pathogen. M-tropic gp120 treatment of APCs reduces their capacity to support T-cell proliferation. As discussed above this may be due to gp120's ability to induce TGF-β1 and prostaglandin release. Prostaglandin, whether added directly, in seminal plasma or induced by gp120, caused an up-regulation of Notch ligand mRNA (chapter 5), and may have acted to reduce T-cell proliferation by signalling through the Notch pathway. Changes in antigen processing and presentation efficiency caused by surface CD4 internalisation, TGF-β, PGE_2 and induction of Notch ligands may all contribute to the reduced capacity of gp120-treated APCs to support T-cell proliferation. Changes in antigen processing and presentation efficiencies caused by CD4 loss could account for the gp120 strain specificity of the proliferation defect, but not the abolition of the defect by indomethacin treatment. The action of indomethacin suggests a role for APC-derived PG in reduced T-cell proliferation. Indeed gp120 induces PGE_2 release by APCs, and direct PGE_2 treatment of APCs causes a reduction in their ability to support T-cell proliferation. PGE_2 may act to reduce T-cell proliferation via its induction of Notch ligands on APCs. It seems likely that all of the signals discussed above will influence the extent to which gp120-treated APCs (and perhaps APCs in general) will drive T-cell proliferation.

There is data emerging to show that the Notch pathway can interact with more 'tradition' immunologic signals. Chapter 5 shows an interaction between the Notch pathway and PG, and other workers have demonstrated that TGF-β and IL-10 act individually and in synergy to induce Notch ligands on murine APC (Lynn Forsyth and Gerry Hoyne, unpublished observations). Alternatively APC-expressed Notch ligands may simply provide T-cells with APC-derived survival signals, so that they stays alive long enough to be influenced by other signals such as cytokines.

Of course, it is possible that gp120-induced PGE_2 may be directly immunosuppressive / tolerogenic, and that a role for Notch does not need to be proposed in order to explain gp120-induced proliferation deficits. However, the PGE_2 concentrations induced by gp120 in cell culture and *in vivo* in HIV-1 infection are not likely to approach the extremely high PGE_2 levels responsible for the immunosuppression caused by seminal plasma (Kelly, 1994; Kelly, 1997). Lower PGE_2 concentrations are likely to induce a Th1 to Th2 switch (Clerici and Shearer, 1993) rather than immunosuppression or tolerance *per se*. Such immune deviation is seen in pregnancy, a state which results in immune modulation (certain autoimmune diseases are aggravated whilst others are abrogated, Koch *et al.*, 1999; Huizinga *et al.*, 1999), but no overall immunosuppression (Kelly, 1994; Piccinni and Romagnani, 2000). A recent review of HIV-1 infection in pregnancy (DeRuiter and Brocklehurst, 1998; Bessinger *et al.*, 1998) concluded that the immune modulation caused by pregnancy in HIV-1^+ women did not change HIV-1 disease progression rates.

New data regarding the distribution of *HES-1* and *deltex* mRNA expression among various cell types is also presented in chapter 5. Whilst lymphocytes and macrophages may be able to transduce Notch / Notch ligand signals via alternative intracellular pathways, only LCs were shown to have the ability to transduce a Notch-mediated tolerisation signal via both HES-1 and deltex (as indicated by changes to *HES-1* and *deltex* mRNA levels). The implications of this finding are unknown but could point to a central role for LCs / DCs in the induction of anergic / regulatory T-cells. With regard to the gp120 induced T-cell proliferation defect reported in chapter 5, it may be that we are looking at a complicated system involving different signals being used to communicate between different cell types. If it were assumed that T-cells do not have the requisite signal transduction

machinery to receive a Notch ligand mediated signal (the absence of *HES-1* and *deltex* mRNA would suggest this), then the role of Notch mediated signalling would be confined to propagating the 'anti-proliferation supporting' phenotype between APCs (monocyte derived LCs appear able to transduce incoming Notch ligand signals via HES-1 and deltex, as evidenced by changes to mRNA levels in response to receiving Notch signals). Cytokines and changes in antigen presentation might be the only signals being fully transduced from APCs to T-cells.

Antigen specific (re)establishment of tolerance is the goal of treatments for autoimmunity and the prevention of graft rejection. Delivery of antigens in an environment that cause the up-regulation of Notch ligands may be sufficient to establish tolerance, or increase the potency of other tolerising signals.
PGE_2, semen and gp120 (or isolated components of the latter two) may all contribute to a tolerogenic environment for antigen presentation by the induction of Notch ligand expression. Although Notch-mediated tolerance of pathogens is deleterious, the induction of Notch ligands by the agents investigated in this thesis could potentially be used to treat autoimmune disease.

Semen and tolerance

It has long been known that PG in seminal plasma establishes an immunosuppressive (and probably a tolerogenic) environment in the female reproductive tract which can prevent an immune response being mounted to allogenic sperm antigens (Kelly and Critchley, 1997b). Changes in IL-10 and IL-12 have been proposed as a mechanism of PG action (Kelly, 1999). Induction of Notch ligand and the immunosuppressive cytokine TGF-β (chapter 5) may also be involved, and contribute to female tolerance of sperm antigens. As an unavoidable consequence of semen's tolerogenicity, inappropriate tolerance to sexually transmitted infections may also appear.

Gp120 mimicking peptides

Chapter 6 describes the production of synthetic peptides, including branched peptides, based on discontinuous gp120 epitopes. These peptides were better able to retain the three-dimensional structure and binding properties of their parent protein

than unbranched linear peptides were. Several of these synthetic peptides were able to block HIV-1_{BAL} infection of macrophages. In addition to this, important observations as to the consequences of peptide stability, and the danger of infection enhancement by gp120-mimics, were made. Since the peptides used in chapter 6 were synthesised by colleagues in the Department of Chemistry new data on the crystallographic structure of gp120 (Kwong *et al.*, 1998) and the regions responsible for determination of viral tropism and chemokine receptor binding (Hoffman and Doms, 1999; Verrier *et al.*, 1999) have emerged. This information will allow the design of future generations of peptides better able to mimic gp120. Several novel therapeutic approaches to HIV-1 disease could be made with gp120-mimicking peptides. In addition to use as vaccines and infection-blocking drugs, gp120-mimics could be used to investigate and combat the gp120-induced immune dysregulation uncovered by work presented here. There is a danger that peptides, such as 4.3 and 3.7, engineered to bind to both CD4 and a chemokine receptor, could cause sufficient cross-linking of cell surface molecules to lead to a macrophage surface CD4 loss similar to that described in chapter 4. Whether this CD4 loss would be damaging (by inducing APC dysregulation and altering presentation efficiencies) or beneficial (by reducing infection of APCs by down-regulating HIV-1's cellular receptors) is not known. Preliminary studies (data not shown) suggest that 3.7 does not cause macrophage surface CD4 loss to the extent seen with R5-tropic gp120. Peptide engineering technology could conceivably be used to link antigenic vaccine epitopes to motifs designed to bind to APC surface receptors (CD4, mannose receptor, Fc receptor) in order to trigger receptor-mediated phagocytosis of the peptide and increased presentation efficiency of the vaccine epitopes. If the induction of Notch ligands by gp120 induces inappropriate *in vivo* tolerance, therapeutic peptides could be used to block gp120 binding and Notch ligand induction. 3.7 has been shown to block gp120 induced T-cell apoptosis by disrupting CD4 / gp120 interactions (Howie *et al.*, 1998). However, 3.7 induced an up-regulation of *delta-1* mRNA in LCs in a similar way to gp120, so may be inappropriate for use in this context.

Peptide antigenicity

It is likely that at least some of the gp120 mimicking peptides in chapter 6 are antigenic in humans, although HLA-restriction will greatly influence individual immune responses. The desirability of this depends on the type of therapeutic intervention being contemplated for the peptides. Obviously, a vaccine candidate needs to be antigenic. For other therapeutic uses (e.g., receptor blockade) peptide antigenicity may be disadvantageous, especially if the peptide is to be given in repeated doses. If an antibody response is mounted to a therapeutic peptide, there is the danger that its properties will be neutralised. A synthetic approach to peptide engineering allows antigenicity to be manipulated with precision. It ought to be possible to closely target immune responses either towards, or away from, defined epitopes. A combinatorial chemistry approach would allow the quick and easy generation of a large library of peptides for efficacy and antigenicity testing. Such an approach might be successful in producing peptides retaining biological (infection blocking) activity but lacking antigenicity, or vaccine peptides capable of inducing an immune response to neutralising epitopes only.

Conclusions

HIV and AIDS have been the subjects of unprecedented research intensity over almost 20 years. A vaccine or cure for AIDS may still be many years away. The research presented in this thesis serves to remind us that despite containing only nine genes, HIV-1 and its interaction with the human immune system is extremely complicated. However, there is cause for optimism in HIV research because new avenues of research are appearing all the time. Data presented here uncover new ways in which HIV-1 can interact with the immune system via its envelope glycoprotein. The cellular tropism of the envelope glycoprotein is shown to be vital in determining its uptake by antigen presenting cells. Functional consequences of gp120 exposure and uptake have also been explored.

The Notch signalling pathway, recently shown to be involved in tolerance induction, is also influenced by gp120. The precise importance of gp120-induced up-

regulation of Notch ligands, the mechanism by which this leads to immune defects, and the interaction between Notch signals and those mediated by more 'traditional' molecules is poorly understood. However, I believe that this thesis presents the first evidence for the possible involvement of Notch signalling in any infectious disease.

Once an understanding of both the molecular interactions of gp120 with APCs, and the functional consequences of these interactions has been gained, the next step forward is to develop the capability to modulate these interactions to therapeutic ends. Recent advances in branched peptide synthesis methods allow the production of peptides that are able to retain a greater amount of the three-dimensional structure of their parent proteins. The three dimensional structure of a region of gp120 was recreated by combining discontinuous gp120 regions around a cysteine-cysteine bond in a branched synthetic peptide. The manufacture of GC-1 and 3.7 were the first applications of this new approach to peptide synthesis. Data showing the HIV-1 infection-blocking properties of 3.7 presented in this thesis are the first to show that peptides made in this way can retain a biological function of their parent protein. If branched synthetic discontinuous-epitope-containing peptides can be made with non-natural amino acids or other modifications to increase their bio-availability, or linked to motifs causing increased phagocytosis or abrogation or increases in Notch signalling, they represent a new class of therapeutics which can be adapted to a range of molecular targets, ever expanding as we enter the post-genomics age.

Insights into human immunology gained by HIV-1 research may have significance that stretches beyond the current AIDS epidemic. The 20th century has seen the emergence of HIV-1, HIV-2, HTLV-I, HTLV-II, Lassa fever, Muerto Canyon virus, and Ebola (Garrett, 1994). Increased immunologic knowledge and new weapons to manipulate the immune response may help mankind outwit more quickly the new viral diseases that will inevitably appear in the 21st century.

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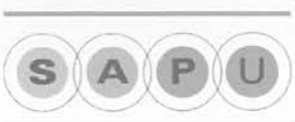

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APPENDIX 1

REAGENT RECIPES

All solutions were made up in distilled water. Recipes are listed in the order they are used in Materials and Methods (Chapter 2). All reagents were obtained from Sigma. Phosphate buffered saline (PBS) was made in distilled water from tablets obtained from Sigma.

Flow cytometry methods

Preparation of monoclonal antibodies

20mM pH 7.3 phosphate buffer (400ml)	112ml 0.02M NaH_2PO_4 288ml 0.02M Na_2HPO_4
0.1M pH2.5 glycine / HCl buffer (1l)	500ml 0.2M glycine 500ml 0.2M HCl
Coomassie blue staining mixture (100ml)	10ml methanol 7ml glacial ethanoic acid 0.25g Coomassie blue
Destain mixture (100ml)	10ml methanol 7ml glacial ethanoic acid

Lowry protein assay

BSA protein standards (200µl each) made up in triplicate in 5ml tubes to give 0, 25, 50, 75, 100, 200, 400 µg ml^{-1}	stock 1mg/ml diluted with 0.1M NaOH
Alkaline carbonate solution (500ml)	5ml 5% (w/v) $CuSO_4.5H_2O$ 5ml 2% (w/v) sodium potassium tartrate tetra-hydrate 20g $NaHCO_3$ 4g NaOH

Phenotyping of cells

Flow buffer	PBS 1% (w/v) BSA 0.05% (w/v) NaN_3
Flow fix	PBS 2% (w/v) formaldehyde

Microscopy methods

FITC conjugation of protein

(All these solutions were filter sterilised)

FITC Labelling Buffer	Make 2 litres, store at 4°C, keep for up to 2 weeks 0.05M H_3BO_3 0.2M NaCl Adjust pH to 7.2 with 5M NaOH
Final Dialysis Buffer	Make 2 litres, store at 4°C 0.1M Tris-HCl buffer pH 7.4 0.2M NaCl Adjust pH to 7.4 with 5M NaOH

Polymerase chain reaction (PCR) methods

DNA gels

10× stock TBE buffer (500ml)	54g tris base 27.5g H_3BO_3 20ml 0.05M EDTA

APPENDIX 2

PCR PRIMER / PROBE SEQUENCE DATA

See Chapter 2 (Materials and Methods) for details of the use of these reagents

Target	GenBank accession	Primer sequences, 5'→3' (S=sense, AS=anti-sense)		T_m / °C	Intron spanning?	Amplicon size / bp	Reference
β-actin	M10277 (genomic) AB004047 (mRNA)	S	TGA CGG GGT CAC CCA CAC TGT GCC CAT CAT CTA	82.1	Yes	661	Innes and Moore (see caption)
		AS	CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG	77.3			
hiv-1	KO3455	S	CTC TAG CAG TGG CGC CCG AAC AGG G [5'LTR]	76.0	Message specific, spans splice site	215	Innes and Moore
		AS	CTA TGA TTA CTA TGG ACC AC [vpu]	46.1			
cd4	M35160 (genomic) M12807 (mRNA)	S	GCA GTG CGG AGC TGT GGT	63.6	Yes	299	Lusso *et al.*, 1993
		AS	GGG TCC CCA CAC CTC ACA GG	67.4			
ccr5 Δ32	U57840 (wildtype) X99393 (Δ32)	S	CAA AAA GAA GGT CTT CAT TAC ACC	58.1	No	190 (wildtype) 158 (mutant)	Innes and Moore
		AS	CCT GTG CCT CTT CTT CTC ATT TCG	65.6			
il-12 p40	M65272 (mRNA)	Primer set purchased from R&D Systems, sequences not disclosed			Yes	559	-
il-10	X78437 (genomic)	S	AAG CTG AGA ACC AAG ACC CAG ACA TCA AGG GG	61.0	Yes	328	Innes and Moore
		AS	AGC TCT CCC AGA GCC CCA GAT CCG ATT TTG G	62.0			

Table A2.1. Details of primers used for conventional PCR. References: Innes and Moore: Primers a gift from Donald Innes and Dr Marilyn Moore, PPL Therapeutics, Roslin, Midlothian, UK. T_m: theoretical primer melting temperature calculated as described in Breslauer *et al.*, 1986.

Target	GenBank accession	Primer / probe sequences (5' → 3') S= sense primer, AS= anti-sense primer, TM= TaqMan® probe)	
delta-1	AF003522.1 (mRNA)	S	GCT TCG TGT GTG ACG AAC ACT A
		AS	AAA GGT CGG TCC CAA GT
		TM	CCG TTT TCT GCC GTC CCC GG
deltex	AF053700 (mRNA)	S	TTC CCT CGC CAC TGC TAT CT
		AS	CTG AGC GGG CAC CAC AAC
		TM	CCC AAC AAC GAG AAA GGC CGG AA
hes-1 / hry	L19314 (genomic) NM_005524 (mRNA)	S	CAT TCT GGA AAT GAC AGT GAA GCA
		AS	CAG CGC AGC CGT CAT CT
		TM	CTC CGG AAC CTG CAG CGG GC
notch-1	M73980 (mRNA)	S	CGG GTC CAC CAG TTT GAA TG
		AS	GTT GTA TTG GGT TCG GCA CCA T
		TM	TGT CCC GGC TGC AGA GCG G
notch-2	X80115 (genomic) U77493.1 (mRNA)	S	TTC CAG TAA TGG TCC AAT ATT TCT T
		AS	CAT CCA CTT CAT ACT CAG AGT TGA CA
		TM	CCT CAT GGC AGT GTG TCC CAG GC
notch-3	NM_000435 (mRNA)	S	GGC TGC AAC ACT GAG GAA TGT
		AS	TCA GGA GGC AGA AGA ACT GTG A
		TM	TGTGCC AGC GAG GTC CCG G
notch-4	GI_4758817 (mRNA)	S	CGG CCT CGG ACT CAG TCA
		AS	GTG TTG AGG TAG GAG TAG
		TM	CGA CGC CGG CCC CAC TAG
serrate-1	U73936 (mRNA)	S	CTT AAC TGT GGC TTG GAT CTG TTG
		AS	GTC CTC AGA GGC TGA GTG TGT GT
		TM	ACT GGT GCC TGC GGA AGC GG
serrate-2	AF029778 (mRNA)	S	CGC TGT ATG AAA GGA GAG AGC AA
		AS	ACT CCT TAT TTT CCT TCT AAG ACA TTT GT
		TM	TCT GCG TCG TCA CCA AAT CGT AGC G
β-actin	M10277 (genomic) AB004047 (mRNA)	Primer set purchased from PE Biosystems, sequences not disclosed	

Table A2.2. Details of primers and TaqMan® probes used for real time PCR. The melting temperature (T_m) was 60°C for all sense primers, and 58°C for all anti-sense primers. All TaqMan probes were FAM conjugated and had a melting temperature of 70°C.

APPENDIX 3

PUBLISHED PAPERS

The following published papers have arisen out of the work presented in this thesis. They are included with the permission the joint authors and the publishers of the journals in which they first appeared. Copyright of the following papers remains vested with these publishers as detailed below. Any licence to copy the main text of the thesis does not extend to this appendix which may not be reproduced except with the written permission of the publishers or in accordance with the provisions of the Copyright, Designs and Patents Act 1988, or under the terms of any licence permitting limited copying issued by the Copyright Licensing Agency Limited, 90 Tottenham Court Road, London W1P 9HE.

Howie, S.E.M.; Fernandes, M.L.; Heslop, I.; Hewson, T.J.; Cotton, G.J.; Moore, M.J.; Innes, D.; Ramage, R. and Harrison, D.J.(1999) A functional, discontinuous HIV-1 gp120 C3/C4 domain-derived, branched, synthetic peptide that binds to CD4 and inhibits MIP-1 alpha chemokine binding. *FASEB J.* **13**, 503-511

Hewson, T.J., Lone, N., Moore, M.J., Howie, S.E.M. (1999) Interactions of HIV-1 with Antigen Presenting Cells (APCs) *Immunol. Cell Biol.* **77**, 289-303

A functional, discontinuous HIV-1 gp120 C3/C4 domain-derived, branched, synthetic peptide that binds to CD4 and inhibits MIP-1α chemokine binding

SARAH E. M. HOWIE,*,[1] MARK L. FERNANDES,* IAN HESLOP,*,† TIM J. HEWSON,* GRAHAM J. COTTON,*,† MARILYN J. MOORE,*,‡ DONALD INNES,*,‡ ROBERT RAMAGE,† AND DAVID J. HARRISON*

Department of *Pathology, †Centre for Protein Technology, and ‡Centre for HIV Research, University of Edinburgh, U.K.

ABSTRACT **This paper describes a branched synthetic peptide [3.7] that incorporates sequence discontinuous residues of HIV-1 gp120 constant regions. The approach was to bring together residues of gp120 known to interact with human cell membranes such that the peptide could fold to mimic the native molecule. The peptide incorporates elements of both the conserved CD4 and CCR5 binding sites. The 3.7 peptide, which cannot be produced by conventional genetic engineering methods, is recognized by antiserum raised to native gp120. The peptide also binds to CD4 and competitively inhibits binding of QS4120 an antibody directed against the CDR2 region of CD4. When preincubated with the CD4+ve MM6 macrophage cell line, which expresses mRNA for the CCR3 and CCR5 chemokine receptors, both 3.7 and gp120 inhibit binding of the chemokine MIP-1α. The peptide also inhibits infection of primary macrophages by M-tropic HIV-1. Thus, 3.7 is a prototype candidate peptide for a vaccine against HIV-1 and represents a novel approach to the rational design of peptides that can mimic complex sequence discontinuous ligand binding sites of clinically relevant proteins.—Howie, S. E. M., Fernandes, M. L., Heslop, I., Hewson, T. J., Cotton, G. J., Moore, M. J., Innes, D., Ramage, R., Harrison, D. J. A discontinuous HIV-1 gp120 C3/C4 domain-derived, branched, synthetic peptide that binds to CD4 and inhibits MIP-1α chemokine binding. *FASEB J.* 13, 503–511 (1999)**

Key Words: antibody · macrophage · PBMC · monoclonal antibody · CDR2 region

A VACCINE AGAINST TRANSMITTED HIV-1 would ideally prevent binding of macrophage (M)[2] -tropic gp120 to CD4 and to *beta*-chemokine binding coreceptor, both of which are required for efficient infection (1–4). Several *beta*-chemokine receptors have been described as coreceptors for primary M-tropic or dual M and T lymphocyte (T) -tropic isolates, including CCR3 and CCR5 (3, 4), whereas adapted T-tropic only strains preferentially use the *alpha*-chemokine receptor CXCR4 (5, 6). A vaccine peptide to prevent primary infection would thus minimally contain conserved regions of the gp120 CD4- and *beta*-chemokine receptor binding sites. The CD4 binding site of gp120 contains five discontinuous conserved residues (7, 8). The *beta*-chemokine receptor binding site is not fully characterized, but conserved residues in the C4 region are involved in M-tropism (7) and residues in the conserved region have recently been shown to be involved (9, 10).

Based on the peptide sequence of HIV-1 IIIB gp120, we have previously described the synthesis of a novel 44-mer three-armed, branched peptide [3.7] (11, 12) containing four residues necessary for CD4 binding (Asp-368 and Glu-370 from C3; Trp-427 and Asp-457 from C4), an oxidized Cys-Cys turn based on the disulfide link between Cys-378 and Cys-445, and two residues, Lys-421 and Gln-422, involved in M-tropism and the CCR5 binding site (9, 10). Peptide 3.7 has a unique structure that could not be reproduced by conventional genetic engineering. This rationally designed peptide contains both T and B lymphocyte epitopes, cross reacts with polyclonal anti-gp120 antiserum, binds to the CDR2 region, domain 1 of CD4, and inhibits macrophage inflam-

[1] Correspondence: Department of Pathology, Edinburgh University Medical School, Teviot Place, Edinburgh EH8 9AG, U.K. E-mail: s.e.m.howie@ed.ac.uk

[2] Abbreviations: BSA, bovine serum albumin; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; Ig, immunoglobulin; M, macrophage; MIP-1α, macrophage inflammatory protein-1α; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PE, phycoerythrin; RANTES, regulated on activation, normal T expressed and secreted; T, T lymphocyte; TRITC, rhodamine.

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matory protein-1α (MIP-1α) chemokine binding and infection of primary macrophages by M-tropic HIV-1. Apart from the relevance to HIV-1, this work also represents a generic approach to the rational design and synthesis of complex peptides with functional biological properties.

MATERIALS AND METHODS

Peptides

The synthesis of peptide 3.7 was as described previously (11, 12). Irrelevant peptides FMDV and PSS023 were used as controls in some experiments. FMDV is a 44-mer peptide based on a sequence derived from a different virus (bovine foot and mouth disease virus) and, like 3.7, has a cys-X-cys bond incorporated in its structure. PSS023 is a random linear 34-mer peptide. There is no sequence homology between 3.7 and either FMDV or PSS023. All peptides were synthesized by Albachem, Edinburgh, U.K.

Antibodies

Antipeptide polyclonal mouse serum was raised as described previously (11, 12). The immunoglobulin G (IgG) fraction was purified using protein G-Sepharose (Pharmacia Biotech Ltd., St. Alban's, U.K.) in a 0.7 × 10 cm liquid chromatography column (Sigma, Poole, Dorset, U.K.) according to the manufacturer's protocol. Anti-CD4 monoclonal antibodies (QS4120 and L120) and sheep anti-gp120 serum (ARP411) were supplied by the NIBSC centralized facility for AIDS Reagents supported by EU programme EVA (contract BMH4 97/2515) and the U.K. Medical Research Council; biotinylated-anti-CD4 (MT310), and rhodamine (TRITC) -labeled antimouse immunoglobulin were obtained from Dako Ltd., Cambridge, U.K.; phycoerythrin (PE) -labeled goat antimouse immunoglobulin, horseradish peroxidase-conjugated, and alkaline phosphatase-conjugated-donkey antisheep serum were purchased from Sigma.

ELISA

Unless otherwise stated, all reagents were purchased from Sigma. 96-Well ELISA microtiter plates (Corning-Costar Ltd., High Wycombe, Bucks, U.K.) were coated overnight at 4°C with 3.7 peptide, FMDV peptide, bovine serum albumin (BSA), or baculovirus expressed recombinant gp120 derived from the HIV-1 IIIB strain (EVA607 supplied by the NIBSC centralized facility for AIDS Reagents supported by EU programme EVA (contract BMH4 97/2515) and the U.K. Medical Research Council) (100 μl/well) in 0.1 M carbonate/bicarbonate buffer pH 9.6. The plate was then washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween 20. Wells were blocked with 1% BSA in PBS for 1 h at room temperature. After three additional washes, a 1:500 dilution of sheep anti-gp120 serum (100 μl per well, diluted with 1% BSA in PBS containing 0.05% Tween 20) was added to the wells and incubated for 2 h at room temperature. The plate was again washed; optimal dilutions of secondary antibody (horseradish peroxidase-conjugated or alkaline phosphatase-conjugated donkey antisheep serum) were added (100 μl per well) and incubated for 1 h at room temperature. Unbound conjugate was removed by washing; *o*-phenylenediamine (0.4 mg/ml in phosphate/citrate buffer pH 5.0 containing 0.006% H_2O_2, 100 μl per well) or 3 M *p*-nitrophenyl phosphate (in 0.05 M Na_2CO_3, 0.5 mM $MgCl_2$) was added and the plate was incubated at room temperature. The coloration reaction was measured at 490 nm for *o*-phenylenediamine or at 405 nm for *p*-nitrophenylphosphate using a Dynatech MR5000 microplate reader.

Cell culture

All tissue culture reagents and plastics were purchased from Life Technologies Ltd., Paisley, U.K., unless otherwise stated. The human T lymphocyte-derived cell line H9 was obtained from the European Collection of Animal Cell Cultures, Porton Down, Salisbury, U.K. The human monocyte/macrophage-derived cell line MM6 was the kind gift from Dr. J. A. Ross, Department of Surgery, University of Edinburgh. Cells were passaged in RPMI 1640 medium containing 10% (vol/vol) fetal calf serum (FCS), 2 mM L-glutamine, and antibiotics (50 IU/ml of penicillin, 50 μg/ml of streptomycin) with the addition of 2.5 μg/ml fungizone for H9 cells.

Peripheral blood-derived macrophages were obtained from single donor Buffy-coat preparations obtained from the Scottish National Blood Transfusion Service. Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation over lymphoprep (Nycomed Pharma AS, Oslo, Norway) and washed in PBS. PBMC were plated into a 24-well plate at 5×10^6/well in Iscove's medium containing antibiotics as described above and allowed to adhere to the wells for 1 h. Nonadherent cells were then removed, the wells were washed, and 1 ml Iscove's medium containing antibiotics and 5% heat-inactivated human AB serum (Scottish National Blood Transfusion Service) were added. The cells were cultured overnight and any remaining nonadherent cells were removed. Adherent cells were cultured for another 4 days (at which point they were >95% CD14+ve, MHC II+ve and CD4+ve macrophages by flow cytometry) before infection.

Colocalization of CD4 and 3.7 on the cell surface

MM6 cells were washed three times in PBS, plated in a microtiter plate at a concentration of 3×10^5 cells/well with or without 1 μg/well of 3.7 and incubated on ice for 2 h. The wells were washed with prechilled binding buffer (1 mg/ml GMEM, 10% FCS, 1 mg/ml HEPES in distilled H_2O, pH 7.2) and incubated with or without 25 μl/well purified mouse antipeptide IgG (2.5 μg) on ice for 1 h. The wells were washed as above and bound antipeptide antibody was detected using TRITC-labeled antimouse immunoglobulin. After further incubation on ice for 30 min and at room temperature for 15 min, cells were washed with prechilled buffer (PBS, 1% BSA, 0.05% NaN_3). Biotinylated mouse antihuman-CD4 mAb was then added and the plate incubated for an additional 0 min on ice, washed with flow buffer, and detected with fluorescein isothiocyanate (FITC) -labeled avidin (Sigma). Cells were fixed in 0.4% formaldehyde and then transferred to slides with a single drop of glycerol/PBS before examination under a Zeiss confocal laser scanning microscope.

Flow cytometry

Flow cytometric analysis was carried out using a Coulter EPICS XL Flow Cytometer (Beckmann-Coulter Electronics, Luton, U.K.) with a 15 mW, single argon ion laser operating at wavelength 488 nm. FITC and PE fluorescence were detected depending on the individual experiment. The percentage of positive cells was established relative to background fluorescence of cells treated with FITC-labeled avidin or PE-labeled goat antimouse immunoglobulin only. Relative

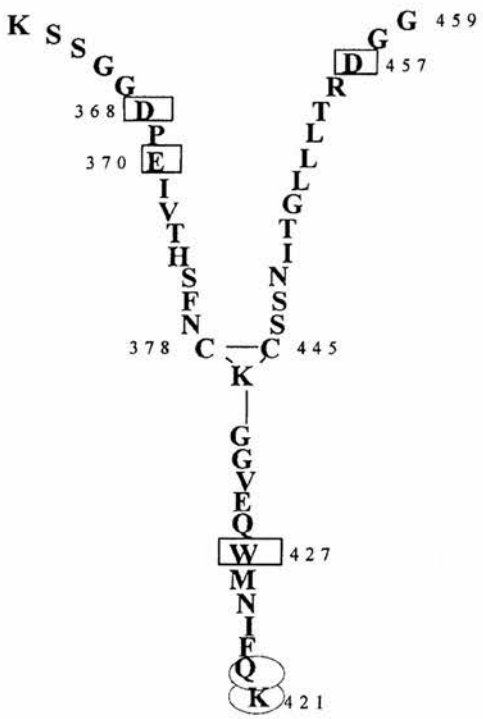

Figure 1. Schematic representation of 3.7. Boxed residues (D-368, E-370, W-427, and D-457) are critical for CD4 binding. Circled residues (K-421 and Q-422) are involved in M-tropism.

intensities of cell surface staining were determined by comparing the mean fluorescence intensity of cell staining within individual experiments.

Anti-CD4 mAb binding

To detect anti-CD4 mAb binding, viable H9 T cells were isolated by gradient centrifugation on Lymphoprep (Nycomed Pharma AS, Oslo, Norway) at 1000 × *g* for 25 min and washed three times in PBS. Cells were then pelleted in a 96-well microtiter plate at a concentration of 10^5 cells/well and 10 μl of 20 nM peptide was added. After incubation for 2 h on ice, 10 μl containing 1 μg anti-CD4 mAb was added. After further incubation on ice for 1 h, wells were washed with prechilled flow buffer (PBS, 1% BSA, 0.05% NaN_3) and bound mAb was detected with PE-labeled goat antimouse immunoglobulin (Dako Ltd.). After incubation for 1 h on ice, the wells were washed in prechilled flow buffer and resuspended in 400 μl prechilled flow buffer in scintillation tubes for analysis.

MIP-1α binding

Binding of biotinylated-recombinant MIP-1α (R&D Systems Europe Ltd., Abingdon, U.K.) to MM6 cells was analyzed after the manufacturer's protocol. Briefly, viable cells were washed three times in PBS, pelleted in a 96-well microtiter plate at 10^5 cells/well, and incubated with or without peptide in a total volume of 10 μl PBS with 1% BSA for 2 h on ice. The cells were then treated with biotinylated MIP-1α, which was detected using FITC-labeled avidin.

Inhibition of HIV-1 BAL infection of primary macrophage cultures

Medium was removed from wells containing adherent macrophages, spun to remove any nonadherent cells and debris, and reserved. Peptide or gp120 was added to quadruplicate wells in 100 μl PBS. The irrelevant random peptide PSS023 and 3.7 were added at 30 μM and recombinant gp120 from the M-tropic MN strain (supplied by the NIBSC centralized facility for AIDS reagents supported by EU programme EVA (contract BMH4 97/2515) and the U.K. Medical Research Council) was added at 0.3 μM. Only PBS was added to control cells. The cells were then incubated for 1 h at 37°C. A previously titrated amount of HIV-1 BAL supernatant (150 μl) was then added to each well such that virus specific message would be detected 72 h after exposure of untreated primary macrophages. Cells were then incubated for 30 min at 37°C. After this time, 750 μl of the reserved culture medium was added to each well and the cells were incubated at 37°C in 5% CO_2 in a humidified incubator.

RT-PCR

Chemokine expression

Total RNA was isolated (Stratagene, Cambridge, U.K.) and 3 μg of RNA was used for cDNA synthesis using Expand reverse transcriptase, 5× RT buffer, DTT (Boehringer Mannheim,

TABLE 1. *Cross-reaction between polyclonal sheep anti-gp120 serum [ARP411] [1:500 dilution] and peptide 3.7 but not the irrelevant peptide FMDV or BSA*

Wells coated with (n = 4)	OD exp. 1 [HRP]	OD exp. 2 [alk phos]	OD exp. 3 [alk phos]
BSA 0.1 μM	0.034 ± 0.005	0.158 ± 0.050	0.153 ± 0.041
gp120 0.1 μM	0.459 ± 0.02*	1.802 ± 0.010*	>2.0*
gp120 0.01 μM	ND	1.020 ± 0.010*	1.096 ± 0.024*
FMDV 1.0 μM	0.017 ± 0.001	0.196 ± 0.007	0.216 ± 0.029
FMDV 0.1 μM	0.037 ± 0.010	0.087 ± 0.008	0.114 ± 0.020
3.7 1.0 μM	0.268 ± 0.033*	0.809 ± 0.063*	0.793 ± 0.037*
3.7 0.1 μM	0.211 ± 0.010*	0.042 ± 0.012	0.011 ± 0.010

* Significantly different from binding to FMDV at same concentration, $P < 0.02$ (Student's t test).

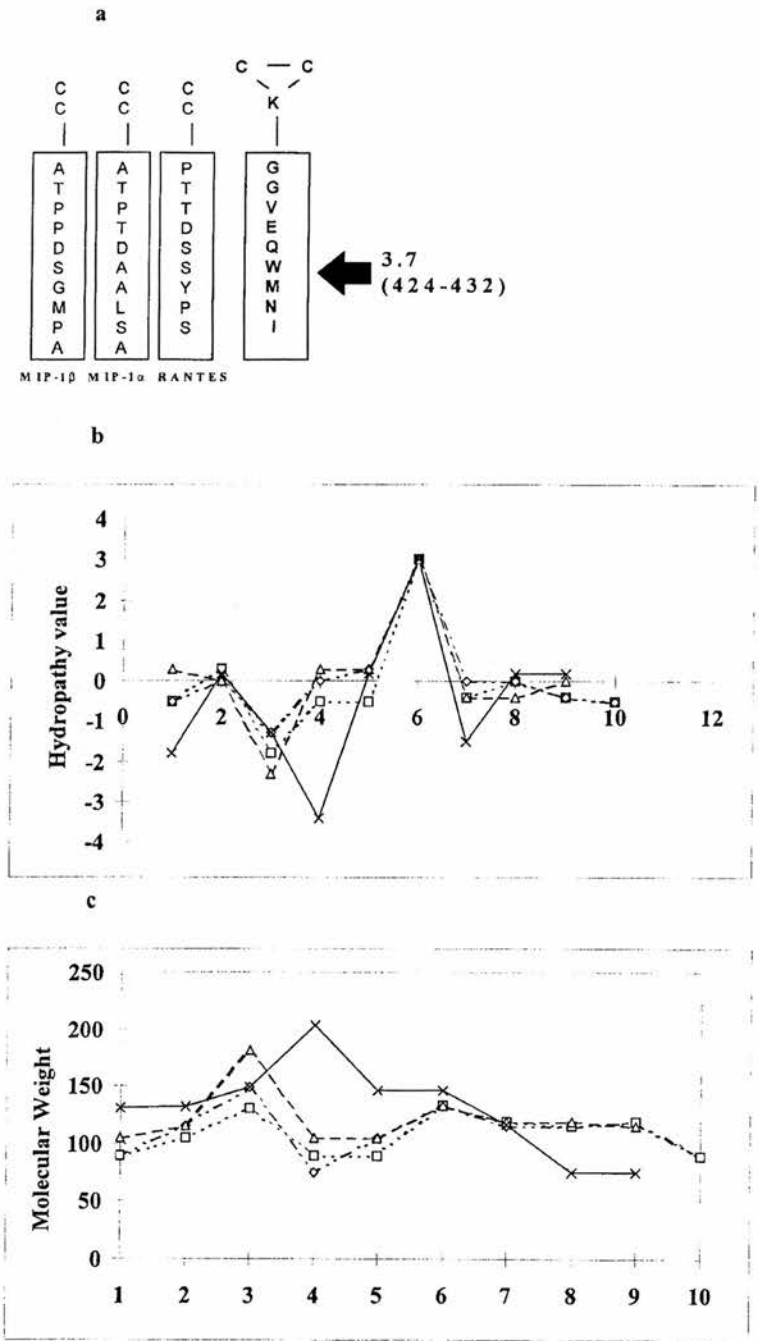

Figure 2. *a*) Sequence comparisons of the receptor binding sites of MIP-1α, MIP-1β, and RANTES (Swiss-Prot database) with 3.7 [424-432]. *b*) Hopp and Woods hydropathy value comparison of the receptor binding sites of MIP-1α (◆), MIP-1β (□), and RANTES (△) with 3.7 (424-432, x). *c*) Molecular weight comparison of the receptor binding sites of MIP-1α (°), MIP-1β (□), and RANTES (△) with 3.7 (424-432, x).

Roche Diagnostics Ltd., Lewes, U.K.), and oligo (dt) (Oswel Ltd., Southampton, U.K.). Products of this reaction were used as a template for polymerase chain reaction (PCR) amplification with Taq DNA polymerase (Promega, Southampton, U.K.) and primers (Oswel Ltd.): CCR5: antisense - CTC GGA TCC GGT GGA ACA AGA TGG ATT AT, sense - CTC GTC GAC ATG TGC ACA ACT CTG ACT G; CCR3: antisense - CCG CTC GAG CAG ACC TAA AAC ACA ATA GAG AGT TCC, sense - CGC GGA TCC GGG AGA AGT GAA ATG ACA ACC; CXCR4: antisense - CCG CTC GAG CAT CTG TGT TAG CTG GAG TGA AAA C, sense - CGC GGA TCC GCG GTT ACC ATG GAG GGG ATC; β-actin: antisense - CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG, sense-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA.

HIV infection

At 72 and 96 h postinfection total RNA was extracted from duplicate wells using the Qiagen RNeasy spin column kit as per the manufacturer's instructions (Qiagen Ltd., Crawley, U.K.). The extracted RNA samples were each treated with 10 units DNaseI (Pharmacia Biotech Ltd.) for 30 min at room temperature. DNase was inactivated by addition of EDTA and incubation at 65 °C for 10 min. RNA content of the samples

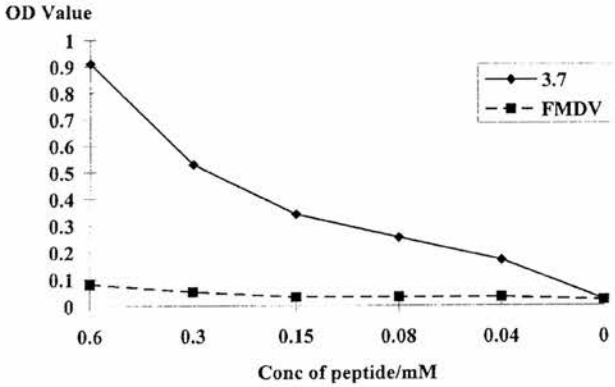

Figure 3. Binding of purified (protein-G affinity binding) murine anti-3.7-IgG to 3.7 and an irrelevant peptide FMDV.

was measured on a GeneQuant (Pharmacia, Biotech Ltd.). RNA (0.1 μg) was used for cDNA synthesis using Expand reverse transcriptase, 5× RT buffer, DTT (Boehringer Mannheim, Roche Diagnostics Ltd.), and Oligo (dt) (Oswel Ltd.). Products of this reaction were used as a template for PCR amplification with Taq DNA polymerase (Helena Bioscience Ltd., Sunderland, U.K.) and primers (Oswel Ltd.) designed from the HIV-1/HTLVIII reference genome sequence, Genbank accession number KO3455, - antisense (vpu) – CTA TGA TTA CTA TGG ACC AC; sense [5'LTR] – CTC TAG CAG TGG CGC CCG AAC AGG G,

RESULTS

Synthesis/structure

Peptide 3.7 was designed from HIV-IIIB gp120, and the numbering is based on this sequence. Highly conserved sequences from the C3 and C4 regions shared by both T- and M-tropic variants were used. The sequence numbering is based on the SWISS-PROT entries P03376 and P04624 (13, 14). Details of the synthesis and rational design of 3.7 are published elsewhere (12). Briefly, the peptide Gly459-Cys445 was synthesized automatedly using f-moc chemistry, Lys was then manually coupled and derivatized and the automated synthesis restarted with an extra Gly residue, followed by Gly-431–Lys-421. After removal of the Dde group on the Lys residue, adding the Cys-378–Ser-364–Lys sequence completed the peptide. The extra Lys residue was added to Ser-364 to give the option of coupling to a carrier. The Cys Cys bond was oxidized in air to give the completed peptide. The sequence of the branched peptide [3.7] is shown in **Fig. 1**.

From sequence data available in the HIV Molecular Immunology Database, this structure contains human cytotoxic T lymphocyte epitopes and human and murine antibody epitopes. The residues Lys-421 and Gln-422 are conserved in T-, M-, and dual tropic isolates but destroy M-tropism when mutated non-conservatively (7) and have recently been shown to be involved in the CCR5 binding site (9, 10). The peptide 3.7 cross reacts with polyclonal sheep antibody raised against baculovirus expressed gp120 whereas an irrelevant 44 mer peptide with an oxidized Cys-X-Cys turn (FMDV derived from a different organism) does not, indicating that 3.7 contains at least some epitopes present in the native molecule (**Table 1**).

To determine whether there was any basis for potential binding of the sequence Lys-421–Gly-431 to *beta*-chemokine receptor, the sequence was compared against that of binding sites on the *beta*-chemokines MIP1α, MIP1β, and regulated on activation, normal T expressed and secreted (RANTES), the 9-10 NH_2-terminal amino acid residues proximal to the first Cys-Cys residues (15). There was no sequence homology, but Hopp and Woods (16) hydropathy and molecular weight plots showed that Lys-424–Gly-432 was similar to the chemokines in terms of charge and size (**Fig. 2**), such that it might fit within a receptor for these chemokines.

Detection of peptide with mouse immune IgG

BALB/C mice were immunized with four doses of 3.7, as previously described (12), and the IgG fraction of serum purified by protein-G-Sepharose column affinity purification. The peptide induced a specific class-switched IgG antibody response without coupling to a carrier molecule, indicating the presence of both helper T and B lymphocyte epitopes (**Fig. 3**). The purified IgG did not bind to an irrelevant peptide, FMDV, of similar size.

3.7 colocalizes with CD4

Dual immunofluorescence studies with CD4-positive MM6 cells showed that 3.7 bound by antipeptide antibody and detected with TRITC-labeled goat an-

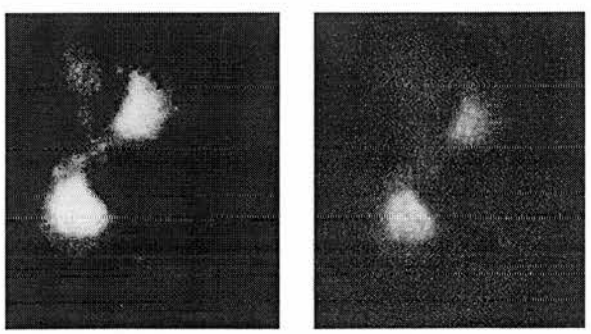

Figure 4. Colocalization of CD4 (*I*) and 3.7 (*ii*) on CD4+ve MM6 cells. Confocal laser scanning photomicrographs at ×1600 original magnification.

timouse immunoglobulin colocalized with biotinylated anti-CD4 monoclonal antibody, detected using FITC labeled avidin (**Fig. 4**).

3.7 binds to the CDR2 region, domain 1 of CD4

To confirm that 3.7 binds to CD4 and to determine to which region it binds, the interaction of different anti-CD4 mAb's with H9 T cells was assessed in the presence of gp120 or 3.7 or an irrelevant peptide of similar size, FMDV. The mAb's used were Q4120 and L120. Q4120 binds to the CDR2 region, domain 1 of CD4, and inhibits gp120 binding to CD4; L120 binds to domain 4 of CD4 and does not inhibit binding of gp120 (12). The mAb's were used at pretitrated concentrations, which gave 30–50% maximal binding to allow inhibition to be detected. Both gp120 and 3.7 inhibited the binding of Q4120 mAb, but not

TABLE 2. *Peptide 3.7 inhibits the binding of QS4120 but not L120 to H9 T cells*

H9 preincubated with	% inhibition of QS4120	% inhibition of L120
3.7 30 μM	22.8 ± 5.3	6.2 ± 3.0
3.7 3 μM	16.2 ± 5.8	1.2 ± 1.2
3.7 1 μM	8.3 ± 0.4	1.0 ± 0.3
3.7 0.1 μM	4.8 ± 0.5	1.0 ± 1.0
FMDV 30 μM	5.6 ± 5.1	6.0 ± 3.1
FMDV 3 μM	2.5 ± 1.5	6.4 ± 4.2
FMDV 1 μM	3.0 ± 1.0	0 ± 0
FMDV 0.1 μM	2.0 ± 1.0	0 ± 0
gp120 0.1 μM	38.0 ± 1.1	0 ± 0
gp120 0.03 μM	23.8 ± 0.6	3.0 ± 0.1
gp120 0.01 μM	6.2 ± 0.8	0 ± 0

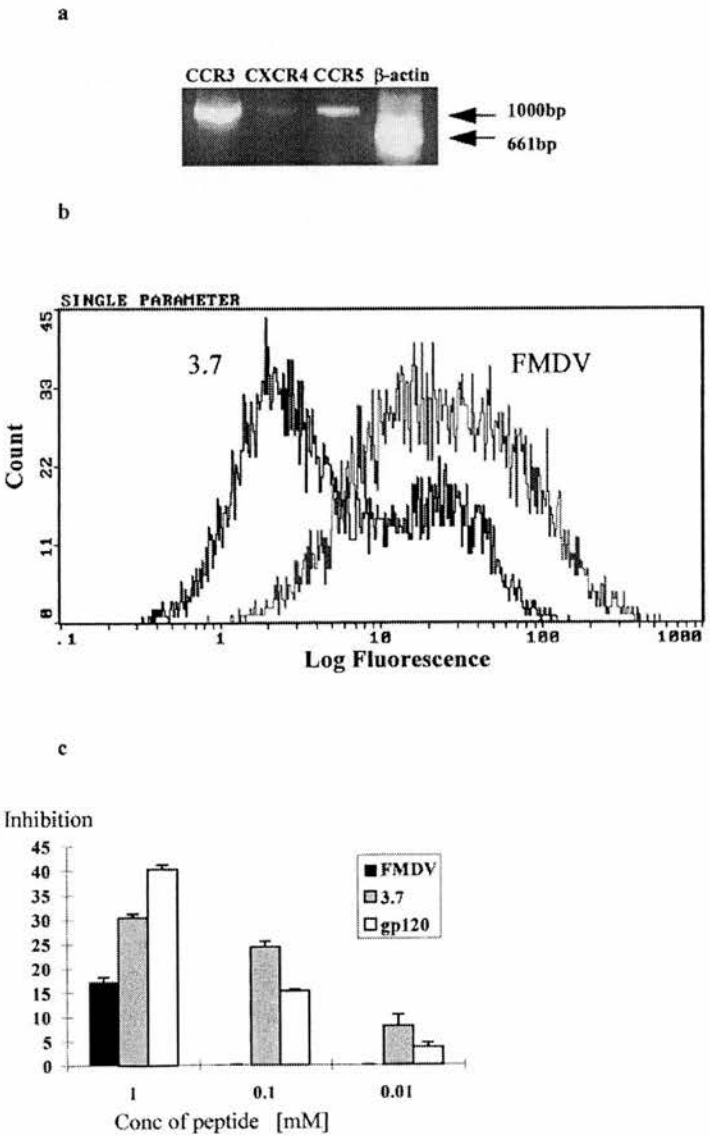

Figure 5. *a*) Expression of chemokine receptors by RT-PCR of MM6 cells. *b*) Inhibition of MIP-1α binding to MM6 cells by peptide 3.7 detected by flow cytometry. Both 3.7 and the irrelevant peptide FMDV were used at 0.1 mM. *c*) Inhibition of MIP-1α binding by peptide 3.7, gp120, and the irrelevant peptide FMDV. FMDV did not inhibit at 0.1 or 0.01 mM. Pooled data from four separate experiments.

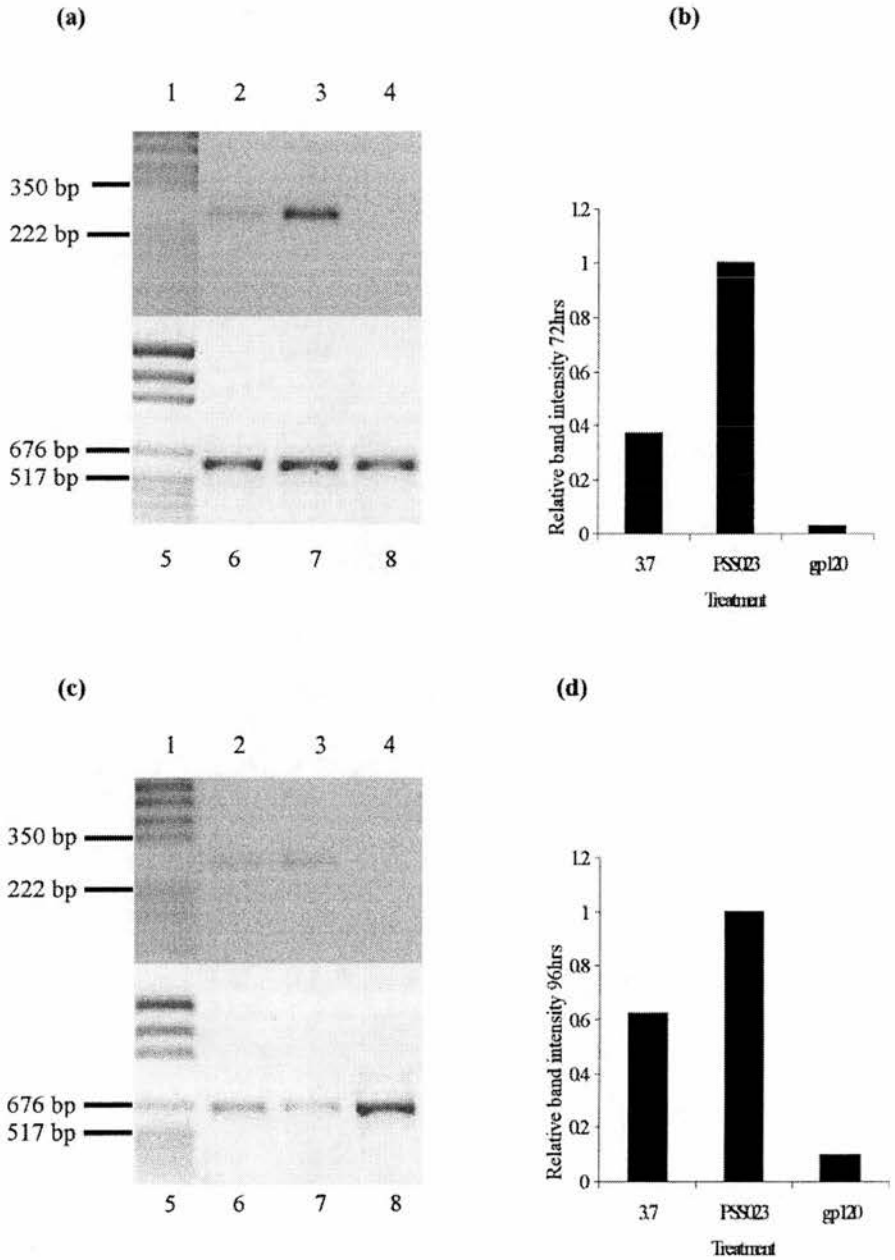

Figure 6. Inhibition of HIV-1 BAL infection of primary macrophages by gp120 and peptide 3.7 after 72 h (*a, b*) and 96 h (*c, d*). *a, c*) 30 cycle RT-PCR showing markers in lanes 1 and 5, RT-PCR with the HIV primers in lanes 2–4, and with the β-actin primers in lanes 6–8. RNA from macrophage cultures preincubated with peptide 3.7 used for lanes 2 and 6, with irrelevant peptide PSS023 for lanes 3 and 7, and with recombinant HIV-1 MN strain gp120 for lanes 4 and 8. *b, d*) Relative densitometry of RT-PCR with HIV-1 primers to RT-PCR with β-actin primers.

L120 mAb, to H9 cells, although gp120 was more efficient on a molar basis (**Table 2**).

3.7 inhibits the binding of MIP-1α to MM6 cells

To investigate the possibility that, like gp120, 3.7, may also interact with chemokine receptors, the ability of 3.7 to inhibit MIP-1α binding to MM6 cells was studied. The macrophage-derived MM6 cell line was selected because these cells are CD4 positive, express CCR3 and CCR5 mRNA (**Fig. 5***a*), and strongly bind recombinant human MIP-1α. Both 3.7 and gp120 significantly inhibited the binding of MIP-1α to MM6 cells whereas the irrelevant peptide FMDV did not inhibit binding (Fig. 5*b,c*)

3.7 inhibits HIV-1 infection of primary macrophages

Since 3.7 bound to both CD4 and chemokine receptors, its effect on the infectivity of the M-tropic HIV-1 BAL strain in primary peripheral blood-derived macrophages was investigated (see **Fig. 6**). Using a semiquantitative reverse transcription (RT) -PCR with β-actin as a reference housekeeping gene, recombinant gp120 blocked infection 72 and 96 h after infection; the irrelevant peptide PSS023 did not block at either time point. The 3.7 peptide markedly inhibited infection on day 3 and to a lesser extent on day 4.

DISCUSSION

The synthetic branched peptide 3.7 cross reacts with native gp120, colocalizes with CD4 on the cell surface, binds to the CDR2 region of domain 1 of CD4, and inhibits MIP-1α binding to H9 cells. The peptide was designed to include four of five residues in the native molecule known to be critical for CD4 binding (7, 8) and the results suggest that the peptide is capable of adopting a structure that allows it to bind to the same region of CD4 as gp120 does. The peptide is less efficient on a molar basis than recombinant gp120, which is not surprising since the percentage of peptide molecules folded in any one particular configuration will be relatively small.

The chemokine receptors CCR3 and CCR5 that bind MIP-1α (18, 19) have been shown to be coreceptors for macrophage tropic HIV-1 gp120 binding (3, 4, 20–22). However, the nature of the chemokine receptor binding site on gp120 is not yet fully understood, although it is known to involve conformational determinants (9, 10) and the V3 loop (23, 24). M-tropism has been shown to involve residues Lys-421 and Gln-422 of the C4 region (7), which were incorporated into the design of 3.7 (12) and have since been shown to be involved in the CCR5 binding site (9, 10). The Hopp and Woods hydropathy values and the molecular weights of residues 424-432 of 3.7 suggested that it might be capable of low affinity binding to receptors for MIP-1α, MIP-1β, and RANTES in addition to CD4. Like gp120, 3.7 did inhibit binding of MIP-1α to MM6 cells, suggesting that the peptide may adopt a structure that allows it to bind to *beta*-chemokine receptors as well as CD4 or that its binding to CD4 causes either a steric alteration or a down-regulation of MIP-1α receptors. We believe it is unlikely that 3.7 signals through CD4 to cause chemokine receptor down-regulation or cytoskeletal changes that render the receptor less accessible to MIP-1α, because all the experiments were conducted on ice. The binding inhibition is not a nonspecific peptide interaction as control irrelevant peptide FMDV had no effect. A number of mechanisms exist by which 3.7 may be inhibiting MIP-1α. First, 3.7 may induce a conformational change in CD4 that causes CD4 to associate with the MIP-1α receptor and, hence, allosterically occlude the MIP-1α binding site. Second, a single molecule of 3.7 may bind to both CD4 and the MIP-1α receptor simultaneously. Third, separate molecules of 3.7 may be binding to MIP-1α receptor and CD4.

Because of the ability of 3.7 to bind to both CD4 and chemokine receptors, we tested its ability to inhibit infection with an M-tropic virus. Using primary, peripheral blood-derived macrophages, we found that 3.7 could indeed inhibit infection with the HIV-1 BAL strain whereas the irrelevant peptide PSS023 had no effect. It may appear paradoxical that a sequence derived from a T cell tropic isolate is able to inhibit ligand binding normally associated with macrophage tropic isolates, but the sequence used is conserved in both T and M tropic isolates. Whereas the V3 loop has been described as necessary for binding to chemokine receptors (6, 24), other regions of gp120 have also been implicated (10, 25–27). It has been reported that the V3 loop interacts with residues from the C4 region (28, 29). Hence, changes in the conformation of the V3 loop may determine whether the residues involved in coreceptor binding from conserved regions of gp120 are in a position that allows interaction of the native molecule with particular coreceptors.

That the synthetic peptide 3.7 derived from three discontinuous sequence stretches of conserved regions can adopt a structure that allows it to interact with cell surface ligands of native gp120 and partially inhibit infection of primary macrophages has implications for the development of both therapeutic intervention and a synthetic vaccine. This approach also has more general implications for the synthesis of novel peptides representing complex, sequence discontinuous ligand binding sites of important biological proteins. FJ

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Review Article

Interactions of HIV-1 with antigen-presenting cells

TIM HEWSON,[1] NAZIR LONE,[1] MARILYN MOORE[2] and SARAH HOWIE[1]

[1]*Department of Pathology, University of Edinburgh Medical School, Edinburgh and* [2]*PPL Therapeutics, Roslin, Mid Lothian, United Kingdom*

Abstract There is currently much interest in the numerical and functional loss of antigen-presenting cells (APC) in HIV-1 disease and the contribution that this may make to HIV-1 pathology. The HIV-1 virus can interfere with the normal function of APC in a number of ways involving inappropriate signalling. These include changes in cytokine balance, cell-surface molecule expression and intracellular signalling pathways. This review examines how HIV-1 is able to disregulate APC function and discusses possible outcomes for the function of the immune system.

Key words: Acquired immunodeficiency syndrome, antigen-presenting cells, dendritic cells, human immunodeficiency virus, immune system, macrophages.

Antigen presenting cell–CD4$^+$ T cell interactions

The interaction between MHC class II-bound antigenic peptides on the surface of antigen presenting cells (APC) and the T cell receptor (TCR) and CD4 on the surface of T helper cells is crucial to the initiation of most antigen-specific immune responses. In addition to TCR interaction with antigen-MHC II on the APC cell surface, the T cell must receive costimulatory signals. If the TCR is triggered in the absence of costimulation, T cells become anergic or undergo apoptosis, mediated through Fas/CD95–Fas ligand interactions.[1–9] T helper 1 cells are reported to be more sensitive to apoptosis via this type of activation than Th2 cells, due to their up-regulation of Fas-ligand expression.[6,10–13] Similarly, if CD4 alone is cross-linked on the T cell surface, either *in vitro*[14,15] or *in vivo*,[16,17] death of the cell results. There are a variety of costimulatory signals requiring cell–cell contact, including ICAM-1/LFA1, CD40/CD40 ligand and CD28/B7, all of which trigger intracellular activation pathways in the T cell.[18–21] There appears to be distinct costimulatory signal requirements for memory and naïve CD4$^+$ T cells.[22–24] In addition to these direct contact interactions, the APC and other local tissue cells (e.g. other leucocytes, stromal cells, epithelial cells, endothelial cells and fibroblasts) release cytokines and other soluble mediators, which also stimulate intracellular activation pathways in the T cell.[25–35] The MHC II$^+$ B lymphocytes, macrophages, dendritic cells (DC) and skin Langerhans cells (LC) all process and present antigenic peptides in cell surface MHC II molecules.[36–42] The type of APC with which the T cell interacts can differ in the cell-surface costimulatory molecules provided and the tissue microenvironment may determine the soluble mediator milieu. The overall balance of these signals determines whether a naïve CD4$^+$ T cell differentiates into a Type 1 or a Type 2 helper cell or whether a memory CD4$^+$ T cell becomes functional.

Correspondence: TJ Hewson, Department of Pathology, The University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG, UK. Email: <t.j.hewson@ed.ac.uk>

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The functional ability of an APC (in common with many other cell types) depends on the cell's lineage, history and the tissue microenvironment. Naïve CD4$^+$ T cells are preferentially activated by mature dendritic cells compared to macrophages and B lymphocytes, while memory T cells can be activated by all three APC types.[23,43] Functional competence of APC can change over time. For example, LC can phagocytose antigen, but lack significant costimulatory activity. When activated by uptake of antigen, they migrate from the skin and travel in the blood as veiled cells to the T cell areas of lymph nodes where they once more change their phenotype, becoming functional DC. Once in the lymph node, they up-regulate accessory molecules and cytokines and lose their phagocytic properties. Thus, during the ontogeny of a DC its functional role changes from that of acquiring antigen, to transporting antigen, to stimulating T-cells.[44–51]

Clinical features of HIV-1 infection

The HIV-1 virus is transmitted by exchange of bodily fluids. The mode of transmission may involve the transfer of free virions or HIV-1 infected cells. Initial (acute) infection with HIV-1 results in clinical symptoms within 1 to 3 weeks in at least half of those newly infected. These symptoms are similar to influenza or mononucleosis along with a non-pruritic macular erythematous rash.[52] Shortly after acute infection, most patients undergo seroconversion. This is followed by a period of clinical latency, which may last from 3 to more than 15 years, before AIDS develops and the patient eventually dies of multiple infections and/or malignancies. Progression to AIDS is accompanied by loss of CD4$^+$ T lymphocytes, with symptoms being noticed at blood levels less than 500 cells/L. Although the vast majority of those who are infected with HIV-1 will develop AIDS, there is mounting evidence that some people are able to live with the virus for extended periods of time without developing clinical disease. Such individuals are termed 'long-term non-progressors', although only time will tell if this group will also succumb to

disease.[53] Factors that affect the rate of progression to AIDS (for review see Levy[54]) include age (most HIV-1 infected infants progress relatively slowly[55,56]), general health (the presence of other infections may speed progression to AIDS[57]) and lifestyle (smoking,[58] alcohol[59] and drug use[60] may all speed progression). Differences in the infecting HIV-1 strain and the host immune response are probably also important in disease progression rates.

Host factors affecting HIV-1 infection and disease progression

Several host factors have been shown to effect progression rate (for review see Roger[61]). As well as providing clinically useful prognostic markers, an understanding of the mechanisms involved in controlling infection and progression rates could be helpful in the search for novel therapeutic approaches.

Genetic factors so far identified as important include chemokine receptor polymorphism (discussed later), HLA polymorphism and less clearly defined host factors that contribute to differential levels of cytokine and chemokine production and immune cell activity (discussed later).

Polymorphisms in HLA and HIV-1 disease progression

The HLA genotype has been shown to influence the time taken for HIV-1 disease to progress to AIDS. Certain alleles or allelic combinations (for example, B27, B51 and (A25 + TAP2.3)) are protective. Other alleles, such as B37 and (B6 + TAP2.1 or TAP2.3) indicate for rapid progression.[62] Polymorphisms in HLA may exert effects through differences in HIV-1 antigen presentation, causing different efficiencies of anti-HIV-1 immune response.[63] Alternatively, because the V3 loop of HIV-1 glycoprotein (gp)120 mimics HLA DR5 and HLA DR6, the anti-HIV-1 TCR repertoire may be influenced by the deletion of self reactive $CD8^+$ T-cells during self-tolerance induction.[64]

Associations of HLA alleles with disease progression rates may be due to genetic linkage between HLA loci and other loci of the MHC, such as those that code for the TNF and complement components. The TNF can induce HIV-1-infected cells to produce virus through activation of NF-κB. Polymorphisms of the TNF promoter have been associated with differential rates of disease progression.[65,66] The complement component, C4, has two null alleles that have been associated with low plasma C4 concentrations, poor antibody responses and rapid progression.[67,68]

Other potentially important host factors

A Danish study has shown an association between homozygosity for loci conferring low serum levels of mannose-binding protein (MBP) and increased HIV susceptibility and shortened survival time between AIDS diagnosis and death.[69]

Many cytokines, especially those that are pro-inflammatory (TNF, IL-1β and IL-6) up-regulate viral replication in infected cells, while other cytokines (Il-4, IL-10 and IFN-β) down-regulate HIV-1 production.[70] It is conceivable that genetic polymorphisms in the inducibility of such cytokines could influence the HIV-1 disease progression rate.

β-Chemokines inhibit macrophage-tropic HIV-1 infection and α-chemokines, such as stromal cell-derived factor (SDF-1), inhibit T cell-tropic HIV-1 infection. A G to A substitution in the promoter of *sdf-1* has been shown to accelerate progression to AIDS.[71,72]

Virus genetic factors affecting HIV-1 disease progression

The lack of HIV-1 disease progression in some long-term survivors (LTS) cannot be attributed to any as yet identified host factor. An alternative explanation for the lack of disease progression is that at least some of the LTS are infected with HIV-1 of a low pathogenicity.[73] Rapid clinical progression is associated with rapid viral replication.[74,75] Defects in the viral genes *nef, vif, vpr, vpu, tat1, rev1, gag* and *env* have all been associated with slowed replication or delayed clinical progression.[73,76–84] A methionine to isoleucine substitution in the initial amino-acyl residue of gag and premature stops have been associated with long-term survival.[73] A G to A nucleotide substitution in the long terminal repeat (LTR) results in a low viral load and long-term survival.[82] A shift from an M- to a T-tropic virus population, as controlled by the sequence of *env*, is associated with disease progression.[75] Long-term survivors have also been identified with an infecting HIV-1 that carries a rare *env* mutation, which renders the virus nearly completely unable to infect $CD4^+$ cell lines, activated PBMC or macrophages.[84]

Human immunodeficiency virus

The HIV-1 virus is a lentivirus of the family Retroviridae. Lentiviruses are all associated with a long incubation period and several are associated with the haematopoietic and immune systems.[54] The HIV-1 has the physical structure characteristic of a lentivirus; this consists of a truncated cone shaped core that contains two copies of a single-stranded RNA genome and the enzyme reverse transcriptase. A protein matrix enclosed in a lipid envelope that bears 72 knobs of the envelope glycoprotein gp160 surrounds the core. gp160 consists of an external gp120 peptide and a gp41 transmembrane component.

Because HIV-1 uses a reverse transcriptase enzyme with a low fidelity,[85] it has a very high mutation rate and consequently much diversity. Viral diversity exists on several levels, but is particularly important in the major immunogenic protein gp120 that has several hypervariable (V) regions.

Clades of HIV-1

The comparison of genomic sequences encoding the V3 region of gp120 has allowed six HIV-1 subtypes (clades) to be identified (A–F, with the O grouping for outlyers and the N (New) grouping from Cameroon).[86] The clades show broad, but distinct geographical ranges. Clade A is prominent in central Africa, clade B in North America and Europe, clade C in South Africa and India, clade D in central Africa, clade E in Thailand and clade F in South America. Such a distribution pattern could be due to patterns of spread, to differences in host immunology brought about by the geographical distribution of selective pressure from other pathogens and/or to differences in the predominant transmission mode. The

HIV-1 epidemic in Thailand is largely due to heterosexual transmission. There is some suggestion[87,88] that the clade E virus, common in Thailand, is especially well suited to this transmission route as it is able to replicate better than clade B viruses in Langerhans cells, which have been implicated in transmission across the vaginal mucosa. Clade B viruses may be better adapted to the homosexual and intravenous transmission routes most common in North America and Europe.

CD4 and HIV-1

The main cell surface receptor for HIV-1 is the CD4 molecule[89] (Fig. 1) and $CD4^+$ T cells are a major cellular target for infection. The gp120 envelope protein on the virion surface binds to CD4 and then to a chemokine receptor (usually CXCR-4 or CCR-5[90]) on the target cell. After CD4 binding, a conformational change in gp120 allows a fusogenic region of gp41 to become exposed and mediate the fusion of the viral envelope and the host membrane. CD4 is an important molecule in the immune system: in addition to acting as an MHC class II coreceptor on T cells, it also acts as a receptor for interleukin-16 (IL-16, originally called lymphocyte chemoattractant factor) on several immune cell types.[91] CD4 is also found on activated cells of the monocyte, macrophage and dendritic lineages; these cells have a role as APC and are infected by HIV-1 both *in vitro* and *in vivo*.

Cellular tropism of HIV-1

The crystal structure of the CD4 binding site has recently been published and this involves several conserved residues in the gp120 molecule.[92] However, it had long been suspected that HIV-1 required an additional, secondary, receptor in order to infect cells. Research since 1995 (for review see D'Souza and Harden[90]) has identified the secondary receptor as being a member of the chemokine receptor family (Fig. 1). At least 10 different chemokine receptors have been identified. The HIV-1 can use several of these molecules as a secondary receptor, but most commonly uses CCR-3, CCR-5 and/or CXCR-4 (for a review of chemokine and chemokine receptor function and nomenclature see Baggiolini *et al.*[93]). The type of chemokine receptor that the virion is able to use depends on the sequence of its gp120. The V3 loop of gp120 is particularly important in determining receptor usage. The viral DNA encoding this region is highly variable between viral strains and also mutates during the course of infection, allowing the virus to change its coreceptor usage, and therefore its phenotype, with time. However, it has recently been shown that conserved regions of gp120 are also important in binding to chemokine receptors.[94] The conformation of the V3 loop changes on CD4 binding, which may allow previously hidden conserved residues access to chemokine receptors.

Because of a differential distribution of chemokine receptors between cell types, the type of receptor that a particular gp120 is able to bind to influences the cellular tropism of the virion. Most primary isolates of virus from patients are macrophage (M)-tropic, can infect both macrophages and T cells and use the CCR-5 coreceptor. Lab-adapted strains grown for many passages on T cell lines only use CXCR-4 as a coreceptor. Patient isolates of primary T cell strains are able to use both CCR-5 and CXCR-4.[95] An alternative classification of HIV-1 tropism refers to the chemokine receptor used

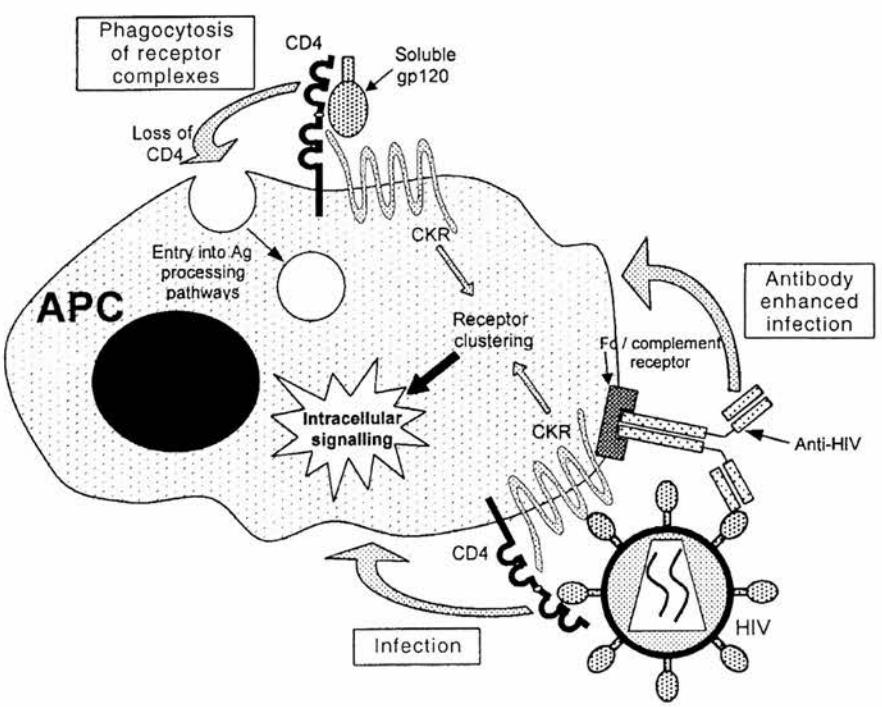

Figure 1 HIV-1 interactions with APC cell surface receptors. HIV-1 and its shed surface protein gp120 are able to interact with a number of receptor molecules on APC. The gp120 protein has binding sites for CD4 and chemokine receptors such as CCR5. It can also interact with Fc or complement receptors via anti-HIV-1 antibodies. Outcomes of HIV-1 proteins binding to APC receptors include infection of the cell by HIV-1 (a process that can be enhanced by antibodies to HIV-1, antibody-enhanced infection), and phagocytosis of receptor complexes bound to soluble gp120, leading to the loss of receptors, such as CD4, and possible entry of the receptor/gp120 complex into antigen processing pathways. It should be noted that all of the HIV-1 receptors are linked to intracellular signalling pathways that can be activated on binding HIV-1 or gp120 and that this can lead to disruption of the cell's function. CKR, chemokine receptor.

as a coreceptor. Under this scheme HIV-1 may be classified as R5 (CCR-5) or X4 (CXCR-4) tropic. It is important to realize that the designation of viral strains to particular tropisms is only an approximation of reality.[96] Tropisms overlap and the infectability of a cell depends on its activation state as well as its phenotype. Almost all HIV-1 strains enter and replicate in activated T cells if added to cultures at sufficiently high concentrations.[95] The HIV-1 virus is able to target a fairly wide range of cell types for infection (see Levy[54] for a comprehensive list), but it may also disrupt the function of other immune cells without infecting them.

Chemokine receptor polymorphisms and disease progression rate

The study of coreceptor usage has led to some interesting clinical observations. *Ccr5* and *ccr2* are both closely linked on chromosome 3p21-22.[97] CCR-5 has three known alleles: wild-type (wt) and two mutations, the *Δ32* deletion[98] and the *m303* premature truncation.[99] Both mutant alleles result in a failure of functional receptor to appear on the cell surface. In populations of European descent, a null mutant in the *CCR-5* gene is present at surprisingly high frequencies: about 18% are heterozygous for a *ccr5* mutation and about 1% are homozygous.[100] The most frequent *ccr5* null mutation is the *Δ32* deletion.

The mutations do not confer a selective disadvantage, but they appear, at least when homozygous, to protect the individual against infection by HIV-1 or to confer a long-term non-progressive disease course.[101] The reasons why such individuals do not become infected by virus using an alternative coreceptor (T-tropic strains using CXCR-4 for example) are obscure. It could be that M-tropic strains (using CCR-5) are responsible for the initial mucosal infection of APC required for sexual transmission of HIV-1.[100]

During asymptomatic HIV-1 infection, the virus is replicating rapidly and with low fidelity.[102] This produces a great deal of diversity in most of the viral proteins, which can allow the virus to evolve resistance to therapeutic drugs. However, during the asymptomatic phase it is usually only possible to isolate CCR-5-using virus. It is only during the symptomatic phase, when the immune system collapses, that broadening of coreceptor usage is seen. There is also a switch from the non-syncytium-inducing (NSI) to the more cytopathic syncytium-inducing (SI) phenotypes. It has been suggested that in the early stages of infection, the immune system suppresses viruses with expanded coreceptor specificity,[100] although the mechanism by which this would be achieved remains obscure.

The extent of protection from infection and disease progression gained by the *wt/Δ32* genotype is controversial. Protection is probably only partial and may only be from transmission by heterosexual sex and not from homosexual and intravenous infection routes.[61,103–105] A *ccr2b* mutation, *64I* (a valine to isoleucine substitution), has also been epidemiologically linked to reduced disease progression rate in HIV-1$^+$ individuals.[61] The amino-acyl residue substitution manifested in the *64I* allele is conservative and found in a transmembrane region of the protein. This observation, together with the fact that HIV-1 rarely uses CCR2b as an important coreceptor, suggests that the *ccr2b64I* allele may not affect disease progression directly, but is merely a linkage marker for another locus that is able to confer protection. One candidate locus to show a linkage to *ccr2b* is the *ccr5* promoter (*ccr5p*). An A/G polymorphism at the *ccr5p* locus has been linked to lower promoter activity and a progression to AIDS 3 or 4 years more slowly than the wild-type *ccr5p*.[106,107] Alternatively, the *ccr2b64I* allele may influence chemokine secretion or CCR-5 or CXCR-4 expression.[108]

Human immunodeficiency virus-1 as an antigen

Infected individuals develop CD8$^+$ cytotoxic T cells that recognize epitopes on a number of HIV-1 proteins, including gp120, and these may control initial infection. The surface glycoprotein gp120, probably because of its exposed position on the virion surface, evokes an especially strong antibody response. However, the anti-gp120 antibody response is ultimately ineffective in controlling HIV-1 infection in most patients, despite the ability of many anti-gp120 antibodies to block infection *in vitro*. gp120 is not only found on the virion surface, but it can also be shed into the extracellular compartment and found in the plasmalemma of infected cells. In the extracellular compartment, gp120 can act as a T cell[109] and B cell[110] superantigen and cause the functional loss of lymphocyte subsets. The anti-gp120 antibody response of most patients is very skewed. Antibodies using the VH3 gene product dominate normal human antibody responses. However, antibody responses to HIV-1 in infected individuals rapidly lose any contribution from VH3 and there is over-representation of the VH4 locus. The deletion of VH3 using B cells is attributed to a gp120 superantigen incorporating sites in the C2 and V4C4 domains of gp120. The exception to this is seen in long-term non-progressors who do have VH3 antibodies present in their serum, but do not make antibodies to the superantigen determinant.

Antigen-presenting cell–HIV-1 interactions

The HIV-1 virus interacts with cells of the immune system in many different ways. Many of the effects of HIV-1 have been principally investigated using T cells or T cell lines, but it has become obvious that the interaction of HIV-1 with cells extends much further than simply infecting CD4$^+$ T cells. It is possible for HIV-1 to target a fairly wide range of cell types for productive infection (see Levy[54] for a comprehensive list), which is a multistage process with possibilities for disregulation of the immune system at every step. In addition, HIV-1 may disrupt the function of, or even kill, other immune cells without infecting them. Extracellular gp120 can also alter the function of APC.

gp120 induces changes in APC cytokine production

Ankel *et al.* have reported that gp120, in the absence of any other viral component, is able to induce interferon (IFN, mainly α with some γ) production in PBMC.[111] The inductive effect of gp120 is abrogated by the addition of soluble CD4 (sCD4) and is dependent on the V3 loop. This suggests that binding and presumably the resultant clustering of CD4 and a chemokine receptor is required and sufficient for IFN production to result.

More recent work[112] has shown that the ability of APC to produce cytokines in response to gp120, and the ability of cells to respond to cytokines, can depend on the differentiation state of the cell. It has been shown that as monocytes differentiate to macrophages they show an enhanced IFN-β production in response to HIV-1 infection, bacterial lipopolysaccharide (LPS) or gp120 treatment. Concomitant to this, the cell sensitivity to IFN, as measured by the induction of protection from vesicular stomatitis virus (VSV), granted by IFN-β increased with differentiation to macrophages because of up-regulation of IFN receptors.

Interleukin-10 secretion in response to gp120 has also been observed, but the level of this did not depend on the cells' differentiation state. Interleukin-10 secretion could cause the switch from Th1 to Th2 helper subtypes observed in HIV-1 disease.[113] The cells only produce IL-12 in response to gp120 if they had been previously primed by IFN-β. Only macrophages could be primed by IFN-β, presumably due to their greater sensitivity to this cytokine.

The effects that the cytokines have on the rest of the immune system and on HIV-1 replication are complex (Fig. 2). Interferon is able to down-regulate HIV-1 expression in macrophages.[114] In contrast, TNF has been shown to up-regulate HIV-1 expression. The exact role of many cytokines, including the novel chemokines, awaits further investigation.

Does HIV-1 induce a Th1 to Th2 switch?

The Th1 to Th2 switch hypothesis, as proposed by Clerici and Shearer, states that progression to AIDS is dependent on a switch from Th1 to Th2 as the dominant Th subset.[113] The evidence to support this assertion includes the observation that in short-term PBMC culture, cells taken from patients of increasing clinical progression show a concomitant increase in IL-4 and IL-10 production and a loss of IL-2 and IFN-γ production. There are also data that show that T cell clones from HIV infected skin biopsies are more likely to be classified as Th2 clones than clones derived from healthy control skin.[115,116] Clerici and Shearer have shown that many HIV-1 exposed, but uninfected, individuals are able to generate strong Th1 type responses and suggest that these individuals are protected from disease by the failure to undergo the normal Th1 to Th2 switch.[113]

The mechanism by which the switch operates may involve T cell- or APC-produced cytokines to bias the Th response to Th2. For example, Ito *et al.* have shown that in PBMC and lung macrophages the HIV-1 protein Tat inhibits production of the Th1 cytokine IL-12.[117]

There is, however, some evidence that argues against the Th1 to Th2 switch hypothesis.[118–120] Romagnani *et al.*[115] have been unable to reproduce the observations by Clerici and Shearer[113] on IFN-γ, IL-1 and IL-4 production in their short-term PBMC culture system. They have argued against using PBMC for such experiments because as well as containing Th cells, PBMC contain monocytes, B cells, NK cells and $CD8^+$ T cells, all of which are capable of producing cytokines. The proportions and absolute numbers of these different cell types will change as disease progresses. Changes in the cytokine profile attributed to a Th subtype switch could be due to selective deletion of Th-cell subtypes. The T cells present in skin biopsies may not reflect the types found elsewhere in the body.

The Th1 to Th2 switch hypothesis has recently become more complicated with evidence that Th2 and Th0 cells are able to replicate HIV-1 more efficiently than Th1 cells.[120,121] Whether this translates into a longer or shorter life span for the infected cells is unclear. Interleukin-4 has been shown to up-regulate and IL-12 to down-regulate CXCR-4 expression and therefore infectability by the T cell-tropic HIV-1 strains associated with disease progression.[122,123] This observation

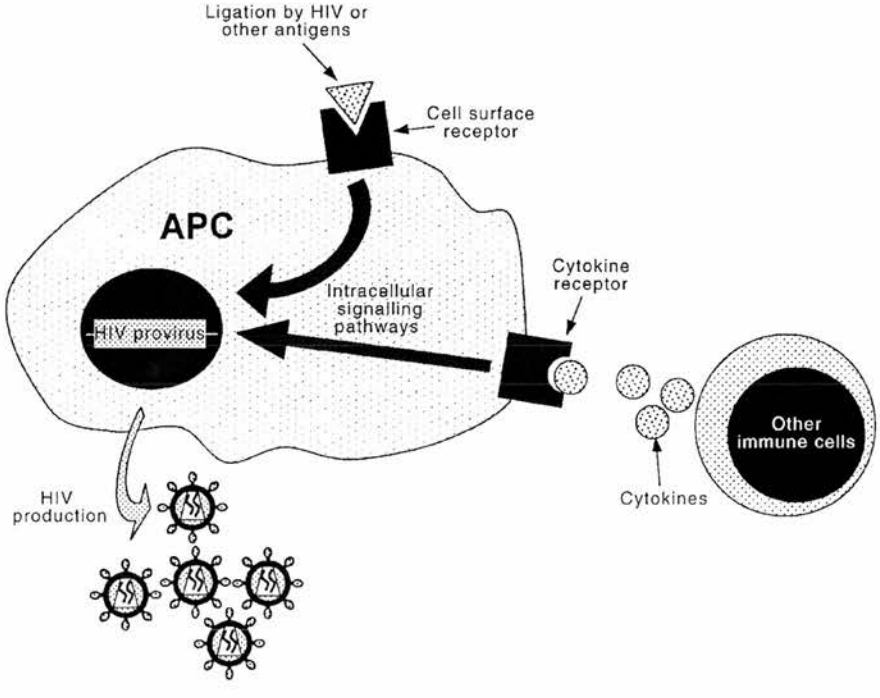

Figure 2 Regulation of virus production by APC. The rate of HIV-1 production by APC is highly influenced by the activation state of the cell. Signalling through cell surface receptors such as CD4 (see Fig. 1) can affect the cellular state. Cytokines produced by other cells in response to HIV-1 or other pathogens also have an influence on HIV-1 production. Interferon and interleukin-10 have been shown to down-regulate HIV-1 production and other cytokines such as TNF may stimulate an increase in HIV-1 production. Finally, the cell's maturation/differentiation state, as influenced by the tissue micro-environment and cytokines, such as granulocyte–macrophage colony-stimulating factor, can alter the cell's susceptibility to infection or the production rate of HIV-1 by an infected cell.

may argue for a Th2 (IL-4) shift increasing selective pressure on the virus to use CXCR-4. However, another Th2 cytokine, IL-10, increases CCR5 (associated with macrophage tropism) expression, so the picture is far from clear.

gp120 induces a cell-signalling defect

Hubert *et al.* showed that in T cells, gp120 is able to cause a dissociation of cell-surface CD4 from cytoplasmic p56LCK.[124] This results in a down-regulation of CD4 expression (a possible mechanism of avoiding HIV-1 superinfection) and an abrogation of signalling through the TCR/CD3 complex. In the context of macrophages, CD4 plays a different role to that in T cells.[91] The p56LCK kinase has never been found in monocytes or macrophages, although other *src* kinases, such as Lyn, are present. It is possible that gp120 can inhibit signalling through CD4 in macrophages, causing CD4/*src* kinase dissociation and reduced CD4 expression.

Work at the Department of Pathology, University of Edinburgh has shown that recombinant purified gp120 is able to induce a substantial loss of CD4 from the surface of cultured primary macrophages.[125] This loss is only observed with gp120 derived from an mRNA sequence from a macrophage tropic primary patient isolate. It was not observed using gp120 from the T cell line-adapted IIIB strain of HIV-1. This strain specificity, together with the failure to observe CD4 loss in *ccr5Δ32* mutant macrophages (effectively *ccr5* null) suggests the involvement of the CCR5 chemokine receptor in gp120-induced CD4 down-regulation on APC (Fig. 1).

gp120 may also interfere more directly with CD4 function by directly competing with its other ligands (MHC class II proteins and IL-16). It has also been suggested,[54] although not proven, that anti-idiotypic antibody mirroring gp120 may have a role in HIV-1 pathogenesis.

Other HIV-1 proteins induce cell-signalling effects

Nef and Vpu are two other HIV-1 proteins that have been shown to induce cell-signalling defects in infected cells. Many activities have been attributed to Nef and the function for which it has evolved is still not fully understood. It is needed to maintain high viral loads[126] and may achieve this by inhibiting the superinfection of cells (this could result in the death of a cell before it had been used to produce many virions[127]) or by optimizing protein sorting to the viral membrane during virus particle assembly.[128]

When the monocyte/macrophage-like cell line, U937, was transfected with *nef*, De *et al.* observed a down-regulation of FcγRI and FcγRII and a changed cytokine response to LPS and PMA.[127] In these myeloid cells, *nef* transfection caused an up-regulation of MHC class I surface expression. In contrast, in T cells Nef induced a down-regulation of MHC class I[129] by causing rapid endocytosis of Nef and MHC class I complexes.[130] Nef has also been demonstrated to induce a CD4 down-regulation, at least in T cells.[131] The HIV-1 protein Vpu is unique among primate lentiviruses in being only found in HIV-1 and the closely related SIV_{CPZ}.[132] Vpu complexes with nascent CD4 in the endoplasmic reticulum and leads to its retention and degradation.[133]

The role of gp120 in inducing leucocyte apoptosis

It has been reported that gp120 interacting with its receptors, in the absence of signalling through the TCR, can lead to T cell anergy and priming of the T cell for activation-induced cell death (AICD), a form of apoptosis, upon receiving a subsequent signal via the TCR.[134] Whether gp120 can prime APC for apoptosis and what trigger would result in cell death is not known.

It has also been reported that HIV-1-infected APC can prime T cells to undergo AICD (Fig. 3).[135] The priming of cells requires two signals from the APC to the T cell to be delivered simultaneously. The first signal is antigen specific and is delivered through the TCR. The second signal is delivered (presumably via CD4 and/or chemokine receptors) by gp120 expressed on the surface of the APC. Experiments involving the transfection of monocytes to express single HIV-1 proteins have shown that gp160 alone is sufficient to constitute the second signal and that no other viral component is required.

Activation-induced cell death can result in hidden damage to the immune system in the absence of a significant decline in T cell numbers. T cell clones, which are required to provide protection against HIV-1 and other pathogens present in the host, are selectively destroyed because it is these T cells that will receive antigen-specific signals from APC.

It may be that HIV-1 infected APC are unable to deliver appropriate cosignals to $CD4^+$ T cells[136] and this may result in T cell anergy or apoptosis. Monocytes/macrophages in HIV-1 disease may produce subnormal levels of IL-12, resulting in T cell death.[137–139] T cell deletion in HIV-1 disease may be the result of an HIV-encoded super-antigen; although this has yet to be identified, some authors have suggested Nef as a candidate.[140,141] There is some evidence that the HIV-1 gene *Tat* can be secreted by infected cells[142,143] and taken up by surrounding non-infected cells.[144–146] Once in cells, Tat can induce oxidative stress by activating NF-κB and TNF-α expression;[147] such oxidative stress can prime cells for apoptosis. Additionally, Tat may be able to directly down-regulate the expression of *bcl-2*, an anti-apoptotic gene.[148]

Other HIV-1 proteins can be cytotoxic in isolation. gp41 is toxic to cells, probably through alterations of membrane permeability.[149] Domains of Nef, Tat and gp41 all show similarities to neurotoxins.[150,151] Nef can change the membrane potential of cells[150] and Tat has been demonstrated to cause neurone death.[152] The effects on other cell types are less well known.

Cell/HIV-1 binding and membrane fusion: Potential for disregulation

As discussed earlier, HIV-1 binds to the surface of target cells by using gp120, which interacts with host CD4 and a chemokine receptor. Although both cellular receptors are usually required for infection, there have been reports of infection of $CD4^-$ T cells.[153] The mechanisms of this are poorly understood and may involve infection of double-positive thymocytes.

The efficiency of infection of APC and other cell types bearing Fc or complement receptors can be enhanced by HIV-1 opsonized for the receptor via a non-neutralizing antibody, a phenomenon known as antibody-dependent

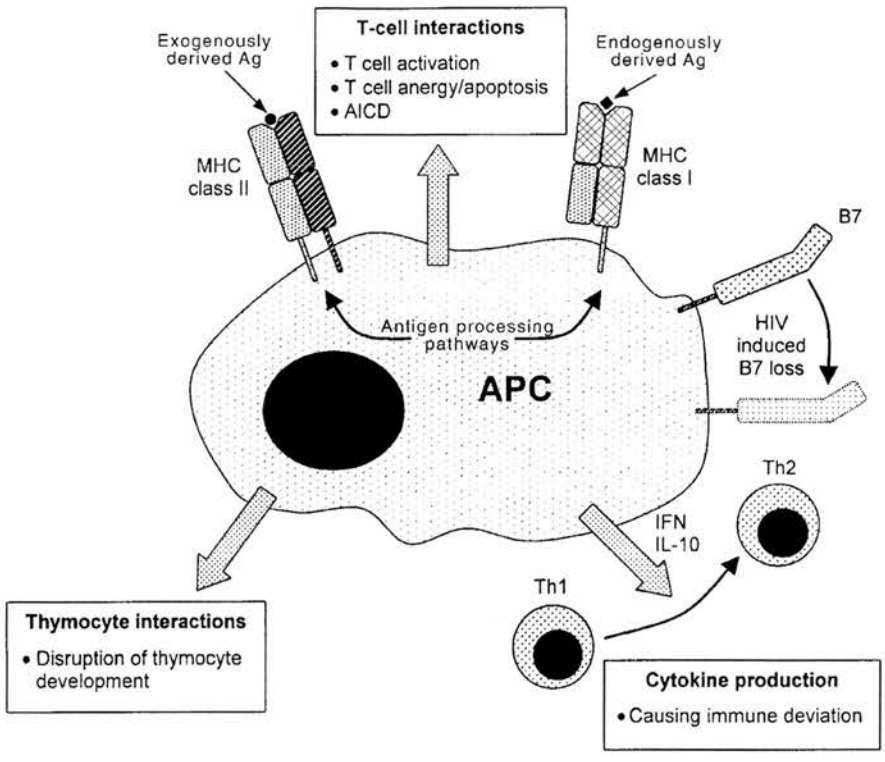

Figure 3 HIV-1 control of antigen presentation and APC–T cell interaction. Anti-HIV-1 T cell responses are observed in HIV disease, so APC must be able, on occasion, to present HIV-1 protein epitopes together with costimulatory signals to T cells. However, HIV-1 infection is able to induce B7 (CD80) loss from the surface of APC; this can result in antigen (from HIV-1 and other pathogens) being presented to T cells in a context that, rather than activating the T cell, can induce T cell anergy or activation-induced cell death. As well as giving inappropriate signals to mature T cells, HIV-1-infected APC can disrupt thymocyte development by inappropriate intrathymic signalling. Antigen-presenting cells infected with HIV-1 can also affect the nature of a T cell response by producing cytokines, such as interferon and interleukin-10, which cause immune deviation from a Th1 to a Th2 response. The Th2 response is less effective at combating HIV-1 disease. AICD, activation-induced cell death.

enhancement (ADE; Fig. 1).[154] In some studies, ADE abrogated the requirement for CD4 binding and allowed the infection of $CD4^-$ cells.[155] In other studies, $CD4^-$ gp120 binding was reported as essential for infection.[156] Ligation of Fc and complement receptors must also have implications with respect to intracellular signalling, regardless of whether infection is the end result.

In some cases, HIV-1 may bind to the surface of a cell in the absence of envelope/plasmalemma fusion and without infection of the cell. However, bound virion may 'piggy-back' on a migrating cell and lead to the dissemination of the virus. Dendritic cells have been implicated in transporting bound virus from the mucosal site of entry to the lymph nodes.[31]

After virion binding, in order for infection to become established, the target plasmalemma must fuse with the viral envelope. Fusion appears to be mediated by a fusogenic portion of gp41.[157] It is important to remember that both the inner and outer lipid monolayers must fuse and that each fusion may be an independent event. It is possible that partial fusion could result with the core unable to enter the cytoplasm. Neutralizing antibodies mostly act by preventing virion binding; some, especially those against gp41, may interfere with the envelope protein's fusogenic function.

The replicative cycle of HIV-1

After cell fusion, the viral core enters the host cytoplasm and the genome and reverse transcriptase molecules are unpacked; the single-stranded RNA genome is reverse transcribed and eventually forms double-stranded DNA. This DNA is then transported to the cell nucleus where it is circularized and integrated into a random site on the host DNA. Non-integrated viral DNA may be able to produce infectious HIV-1,[158] but integration is a requirement for efficient, long-term virion production.

Sun *et al.* have shown that HIV-1 can enter resting $CD4^+$ T cells and that the initiation of reverse transcription can take place in these cells.[159] Formation of the full length viral DNA requires the cell to be activated by TCR ligation (signal one), a signal that normally regulates the G0 to G1 transition. Transport of the viral DNA to the nucleus to allow for integration requires an additional signal (signal two, costimulation via CD28 ligation). The second signal has been shown to be IL-2 receptor dependent and sensitive to cyclosporin A. The signals required to stimulate HIV-1 production in APC may be different from those required by T cells, but the differentiation/activation state of the cell will still be important. For a discussion of the dynamics of HIV-1 replication see elsewhere.[160–162]

Although macrophages express less CD4 than their monocyte precursors, macrophages are more susceptible than monocytes to HIV-1 infection. In contrast to T cells, non-dividing, resting macrophages can become productively infected.[163] However, macrophages produce HIV-1 at a slower rate than do T cells; this may be a result of reduced intracellular pools of nucleotides and other precursors in macrophages, which show slower division rates than T cells.

Granulocyte–macrophage colony-stimulating factor (GM-CSF) has been implicated in some of the signals controlling

HIV-1 production by APC of the monocyte/macrophage lineage. Crowe and Lopez showed that GM-CSF, a cytokine produced by many cell types including activated T cells, can affect the replication of HIV-1 in cells of macrophage lineage at several levels.[164]

Monocyte survival is promoted by GM-CSF through the inhibition of apoptosis and the stimulation of proliferation, which increases the number of HIV-1 targets. The differentiation of monocytes is also promoted by GM-CSF. On its own, GM-CSF stimulates differentiation to macrophages; with IL-4 also present, monocytes are driven to become dendritic-like cells. Differentiation of monocytes down either of these pathways appears to result in the down-regulation of surface CD14,[165] although one study claims that in the monocyte/macrophage cell line U1, GM-CSF up-regulates CD14 expression.[166] Differentiation driven by GM-CSF may be important in HIV-1 disease because it turns monocytes, cells that are relatively resistant to infection, into more likely target cells.

The long-terminal repeat: The HIV-1 promoter

After HIV-1 DNA has been integrated into a host chromosome, the production of new virions requires the transcription, by host transcriptases, of the provirus, to produce both viral mRNA and genomic viral RNA for packaging. Transcription of viral genes is under the control of the long terminal repeat (LTR), a viral promoter found directly 3′ and 5′ to the viral genes. The effects of cellular activation and cytokines on HIV-1 expression can be explained in terms of the transcriptional effects on the LTR.

The initiation of HIV-1 transcription is under the control of cellular factors that bind to the LTR. Nuclear factor-κB, nuclear factor of activated T cells (NFAT), activation protein 1 (AP-1, consisting of a Jun/Fos heterodimer) and Sp-1 have all been shown to activate HIV-1 transcription (for review see Gaynor[167]). Once transcription has begun, the viral protein Tat (transactivator) can interact with the transactivation responsive (TAR) regulatory element and the requirement for cellular transcription factors may be reduced.

The transcription factor NF-κB in resting T cells is sequestered in an inactive form bound to an inhibitory subunit, I-κB. Activation signals received by the cell through the TCR have the downstream effect of phosphorylating I-κB and this allows NF-κB to be released, bind to and activate both host and viral genes. Alcami *et al.* have shown that transcriptional activation by NF-κB is an absolute requirement for HIV-1 transcription and that Tat/TAR mediated amplification can only occur as a result of earlier NF-κB effects.[168]

CD14 levels can also be important in the control of HIV-1 expression. CD14 acts as a receptor for LPS/LPS binding protein complexes and thus mediates responses to LPS.[169] CD14 therefore has a role in mediating LPS-induced up-

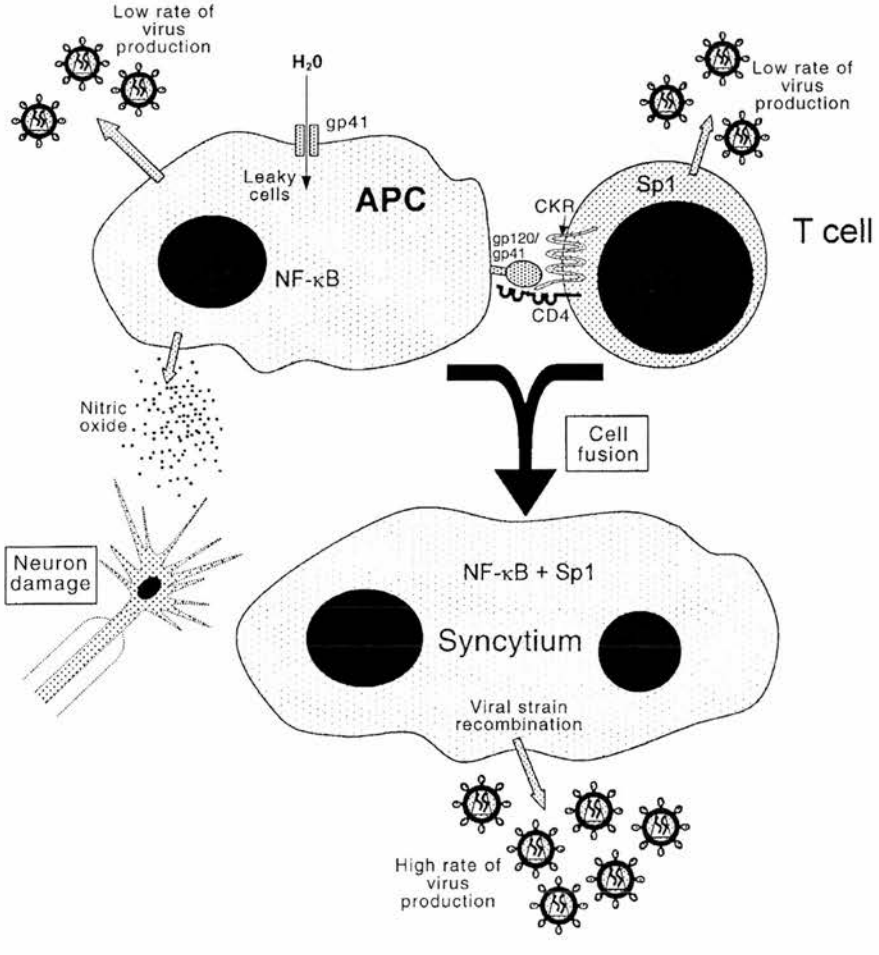

Figure 4 Other HIV-1/APC induced cellular damage. Macrophages infected with HIV-1 have been shown to produce nitric oxide, which is a neurotoxin. The damage done to neurons in this way may contribute to AIDS-related dementia. Immune system damage may result from the HIV-1 proteins gp41 and gp120 leaking onto the plasmalemma of infected cells. In the case of gp41, this can be directly cytotoxic due to the formation of pores in the cell membrane. Leaking of viral proteins to the cell surface can also induce cell–cell fusion in a fashion similar to cell–virion fusion. The resulting syncytium not only results in the loss of the daughter cells, but the cell–cell fusion can be advantageous to the virus, because it brings together nuclear factors important for HIV-1 transcription, such as nuclear factor (NF)-κB and Sp1, in the same cell and this allows for accelerated virus production. Syncytia may also be sites at which interstrain recombination can take place increasing virus genome variability.

regulation of HIV-1 expression in HIV-1 infected monocytes. This up-regulation is abrogated by anti-CD14 antibodies.[166,170]

It has also been shown that GM-CSF has a more direct effect on HIV-1 expression by enhancing *in vitro* virus production in primary monocytes and macrophages, but not in T cells or Langerhans cells.[164]

Thornton *et al.*, in work on the U937 monocytic cell line, presented evidence to suggest that HIV-1 can subvert the normally antiviral interferon response to control viral replication.[153] The HIV-1 has a sequence in its regulatory region mimicking the IFN-stimulated response element (ISRE). The ISRE normally activates transcription of antiviral defence genes in response the binding a member of the IFN regulatory factor (IRF) family. Use of IRF-dependent transcription could be an advantage to HIV-1, because IRF proteins are activated on viral infection.

Syncytia induced by HIV-1: Implications for HIV-1 transcription

An important *in vivo* and *in vitro* feature of HIV-1 is its ability to induce syncytia (multinucleate cells) by the fusion of an infected cell with other infected or uninfected cells (Fig. 4). Cell-to-cell fusion and syncytia formation involves CD4, chemokine receptors, gp120 and gp41 and appears to be similar to cell–virion fusion. Fusion to syncytia may result from gp160 leaking onto the plasmalemma of infected cells and interacting with CD4 and chemokine receptors on other cells. Usually primary macrophage tropic strains, using CCR-5, do not form syncytia in T cell lines, whereas viruses able to utilize CXCR-4 often do. The categorization of strains into syncytium-inducing (SI) and non-SI (NSI) phenotypes is, undoubtedly, an oversimplification, although coreceptor usage must be important.[95]

Burke suggests that cell-to-cell fusion is an important HIV-1 evolutionary strategy, analogous to sex, which facilitates recombination between viral strains by allowing them to come together in a syncytium, a structure likened to a mating ground.[171] From a molecular-biological point of view, syncytium formation may be important in bringing together transcription factors from different cells to allow efficient HIV-1 expression. It has been suggested that syncytium formation could allow HIV-1 replication to take place in non-activated memory T cells.[172] Experiments have shown that NF-κB and Sp-1 are both vital transcription factors for HIV-1. They have suggested that non-activated T cells fail to express HIV-1 because they contain Sp-1 but not NF-κB. Purified dendritic cells fail to support HIV-1 replication because they lack Sp-1, despite containing high levels of NF-κB. Dendritic cell/T cell syncytia bring together the two factors and allow up-regulation of viral transcription in the absence of immune stimulation.[172]

Immune status and HIV-1 replication rate

A recent paper by Wahl and Orenstein reviews work that suggests that the activation state of the host immune system has an important control over HIV-1 replication rates.[57] Evidence is presented to show that vaccination and infection can both activate the immune cells and lead to an increase in viremia. Increased HIV-1 replication under the influence of other microbes can be a result of changes in immune cell activation or of a more direct interaction. For example, early gene-products of human herpes virus can exert an effect on the HIV-1 LTR and enhance HIV-1 expression.[167]

Loss of APC numbers in HIV-1 disease

Human immunodeficiency virus-1 disease leads to a reduction in the number of APC in the periphery (losses in skin, blood and gut have all been described).[173] There are several possible reasons for this loss in cell number.

The loss of cells may be due to the lysis of infected cells by the CTL response. *In vitro,* DC can be targeted by anti-HIV-1 CTL.[173] It may be that the killing of an APC by the CTL that it has just activated is part of a normal negative-feedback mechanism of controlling excessive T cell activation. Such a mechanism would only become a problem in HIV-1 disease, because of the persistence of infection (leading to sustained, long-term loss of APC) and the reduced capacity for APC replacement (see later).

Loss of Langerhans cells from the skin may simply reflect migration of cells from the periphery to the lymph nodes in response to activation. Alternatively, APC may be lost by being fused with T cells during syncytia formation or they may die *in situ* by apoptosis as a direct result of infection.

Levy suggests that host cells could be destroyed in an autoimmune fashion by other immunocytes, which recognize host proteins on these cells as foreign because they are linked to an HIV-1 protein (gp120, for example) acting as a hapten.[54] Antibodies to cellular proteins and a wide range of autoimmune disorders have been detected in HIV-1 infection.[174] Whether APC are targeted in this way is not known.

A reduction in Langerhans cell numbers in skin could be due to a failure of haematopoiesis or a failure of tissue colonization by cells from bone marrow progenitors. Knight and Patterson report that $CD34^+$ bone marrow-derived stem cells show little capacity to develop (morphologically or functionally) into DC in patients with advanced AIDS.[173]

Damage done to the immune system by APC in the presence of HIV-1

As has been previously noted, HIV-1-infected APC or APC that have interacted with HIV-1 proteins show a reduced capacity to stimulate T cell effector function and may even prime T cells for AICD. The decline in stimulatory capacity is probably the result of the loss of immunologically important surface molecules by APC in HIV-1 infection. Gabrilovich *et al.* have shown that infected DC down-regulate MHC class II, CD44 and CD54.[175]

Antigen-presenting cells are not only important in initiating an immune response, but in determining the direction that the response takes. Kuchroo *et al.* suggest that the divergence of the T cell response to the Th1 (cytotoxic) or Th2 (humoral) subtype is controlled by differential expression of B7.1 and B7.2 by APC.[176] A switch from a Th1 (IL-2 and IFN-γ mediated) to a Th2 (IL-4 and IL-10 mediated) response has been suggested as a critical step in HIV-1-disease establishment and progression. This switch is absent in many seronegative HIV-1-exposed ('resistant') individuals, who continue to generate Th1-type responses to HIV-1.[113]

The type of APC and the concentration and type of antigen may be important in fixing the response type.[173] It would be interesting to know if the primary macrophage culture system used in our laboratory in the Department of Pathology to show gp120-induced down-regulation of CD4 could be used to demonstrate a gp120-induced shift in response type (manifested by changing B7.1/B7.2 expression on the macrophage or by changes in cytokine production).

Not only do APC exert an effect on T cells during the induction of an immune response, they are also important regulators of T cell development. Infected DC in the thymus may result in inappropriate signalling to developing thymocytes. This would result in the development of an abnormal T cell repertoire.[173]

In her controversial danger hypothesis, Matzinger suggests a mechanism whereby Langerhans cells in the skin can induce tolerance (i.e. anergy or death) in $CD4^+$ T cells.[177] Her idea is that Langerhans cells in the skin express self-antigen/MHC class II complexes, but not costimulatory molecules. T cells interacting with these Langerhans cells would receive signal one but not signal two and would be tolerized. Matzinger suggests that tissue Langerhans cells may phagocytose and present environmental antigen, but viral antigen may also be presented by Langerhans cells (at the site of initial infection) to virus specific $CD4^+$ T cells and this would result in the deletion or anergy of these T cells.

Of course, it is not only cells of the immune system that are damaged by HIV-1. Bukrinsky *et al.* showed that HIV-1 infection induced macrophages to produce nitric oxide, a molecule implicated in the neurological disease seen in some AIDS patients.[178]

Role of APC in HIV-1 dissemination

Acquired immune deficiency syndrome is a systemic disease with HIV-1 infecting cells throughout the body. After sexual transmission, HIV-1 is initially localized to the point of entry. Antigen-presenting cells have been implicated in allowing the virus to spread throughout the body.

Simian immunodeficiency virus (SIV) infection in rhesus macaques has been used as a model for the early events in heterosexual HIV-1 infection.[179,180] When macaques are inoculated intravaginally with SIV, the virus first appears in, or bound to, DC of the lamina propria of the vaginal mucosa. Within 2 days, infected cells are detectable in the draining lymph nodes and by day 5 the infection becomes systemic with SIV detectable in the blood.[180]

Weissman *et al.* have reported that DC can bind HIV-1 and that once these cells have matured and are expressing co-stimulatory molecules (B7.1, this would be in the lymph node *in vivo*) they can stimulate T cells.[181] The stimulation of the T cells activates them and allows them to support productive infection by the virus passed from the infected DC.

Conclusions

It has been 17 years since AIDS was first identified. Approximately 40 million people are now infected with HIV-1 (1% of the world's sexually active population). There have been some recent advances in treatment (for review see Clumeck and Hermans[182]), which have allowed the life-span of $HIV\text{-}1^+$ patients to be increased. Most of the progress in drug treatment has come from an increased understanding of HIV-1 virology and biochemistry. However, in order to find improved treatments and eventually a cure and to start to think about reconstructing a battered immune system after successful treatment to reduce viral load, a greater understanding of immunology will be required.

The APC appears to play a central role in the immune system, regulating the actions of other cell types. The goal of HIV vaccine development may also be served by a greater understanding of APC interactions with the viral components of a putative vaccine. A successful outcome of vaccination depends on appropriate APC/HIV interactions as the first stage of a protective immune response, rather than APC/HIV interactions acting to cause immune system dysfunction and disruption.

An understanding of APC–HIV-1 interactions may hold the key to many of the remaining mysteries of AIDS.

Acknowledgements

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